TABLE 1 Baseline characteristics and operative data on patients who underwent hepatectomy: data are reported for whole study and for the matched study population after propensity score analysis

	Overall series		*P* value	Propensity-matched series		*P* value
	IFN (+) $n = 43$	IFN (−) $n = 76$		Peg-IFN (+) $n = 38$	IFN (−) $n = 38$	
Age (years)	65 (53–78)	71 (48–83)	0.0003	65.5 (53–75)	69 (51–80)	0.2
Sex (male/female)	27/16	47/29	0.918	23/15	25/13	0.634
Preoperative IFN	24 (55.8%)	29 (38.1%)	0.06	20 (52.6%)	14 (36.8%)	0.16
HCV genotype			0.876			0.6
1b	34	61		29	27	
2b	9	15		9	11	
Diabetes mellitus	11 (25.6%)	22 (28.9%)	0.856	11 (28.9%)	13 (34.2%)	0.621
ECOG PS			0.831			0.644
0	39	68		36	35	
1	4	8		2	3	
Platelet ($104/mm^3$)	10.3 (3.3–26.6)	10.3 (3.8–40.3)	0.381	9.75 (3.3–21.5)	11.2 (3.8–40.3)	0.454
T-Bil (mg/dl)	0.7 (0.3–1.4)	0.8 (0.3–1.7)	0.292	0.7 (0.4–1.4)	0.7 (0.3–1.7)	0.798
AST (IU/l)	42 (18–121)	48 (16–150)	0.152	43.5 (18–127)	41.5 (6–150)	0.567
ALT (IU/l)	38 (13–127)	41.5 (10–196)	0.987	40.5 (11–127)	37.5 (10–196)	0.226
Albumin (g/dl)	3.8 (2.8–5.2)	3.8 (2.5–4.9)	0.215	3.8 (2.8–5.2)	3.8 (2.5–4.5)	0.469
ICGR 15 (%)	17.9 (7.4–77.4)	18.7 (4.6–50.5)	0.734	17.65 (7.4–40.0)	17.55 (4.6–40.0)	0.561
AFP (ng/ml)	11.6 (0.5–3405)	27.6 (0.5–36572)	0.176	13.95 (0.5–3405)	22.9 (0.5–513)	0.635
Child–Pugh grade			0.665			0.556
A	41 (95.3%)	69 (90.8%)		37 (97.4%)	36 (94.7%)	
B	2 (4.7%)	7 (9.2%)		1 (2.6%)	2 (5.3%)	
Hepatic resection			0.322			0.373
Hr0	20 (46.5%)	49 (64.5%)		18 (47.4%)	23 (60.5%)	
HrS	13 (30.2%)	18 (23.7%)		12 (31.6%)	9 (23.7%)	
Hr1	3 (7.0%)	4 (5.3%)		2 (5.3%)	3 (7.9%)	
Hr2	7 (16.3%)	5 (6.6%)		6 (15.8%)	2 (5.3%)	
Hr3	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
Operation time (min)	260 (128–623)	242 (90–580)	0.0514	257 (128–623)	247.5 (90–580)	0.18
Blood loss (ml)	200 (20–1900)	225 (10–960)	0.996	210 (20–1900)	210 (10–960)	0.803
Postoperative complications			0.933			0.798
IIIa	4	6		2	2	
IIIb	1	1		1	1	
IVa	1	1		1	0	
Stage			0.315			0.293
I	14 (32.6%)	19 (25.0%)		13 (34.2%)	9 (23.7%)	
II	18 (41.9%)	44 (57.9%)		15 (39.5%)	23 (60.5%)	
III	9 (20.9%)	12 (15.8%)		9 (23.7%)	6 (15.8%)	
IV-A	2 (4.7%)	1 (1.3%)		1 (2.6%)	0 (0.0%)	
Single tumor	28 (65.1%)	57 (75.0%)	0.252	25 (65.8%)	29 (76.3%)	0.312
Tumor size			0.712			0.589
≥3 cm	15 (34.9%)	24 (31.6%)		10 (26.3%)	8 (21.1%)	
<3 cm	28 (65.1%)	52 (68.4%)		28 (73.7%)	30 (78.9%)	
Vascular invasion	4 (9.3%)	3 (3.9%)	0.233	3 (7.9%)	0 (0.0%)	0.239

Continuous variables expressed as median (range)

Hepatic resection and stage were according to General Rules for the Clinical and pathological Study of Primary Liver Cancer, by Liver cancer Study Group of Japan, 5th edition, Kanehara Co., Ltd

Hr0: limited resection, HrS: segmentectomy, Hr1: sectionectomy, Hr2: hemihepatectomy, Hr3: more than hemihepatectomy

T-Bil total bilirubin, *PS* performance status, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ICGR 15* indocyanine green retention rate at 15 min, *AFP* alpha-fetoprotein,

did not receive IFN therapy was 3.8 (1.2–6.9) and 3.5 (1.3–6.8) years, respectively. In the matched study groups, the 3- and 5-year overall survival rates of patients who received peg-IFN therapy after hepatic resection were significantly higher than those of patients who did not receive IFN therapy ($P = 0.00135$) (Fig. 1c). However, there was no significant difference in disease-free survival between the two matched groups ($P = 0.886$) (Fig. 1d).

In the matched 38 patients of the peg-IFN group, peg-IFN therapy was initiated at a median of 4.3 (0.9–9.6) months after hepatic resection. Thirty-one of 38 HCC patients began peg-IFN therapy within 6 months after hepatectomy. Seven patients required more than 6 months to commence peg-IFN therapy. Two patients required a longer time to recover platelet counts of more than 70,000/μl. Five patients required a longer time to decide to receive peg-IFN therapy. Sixteen (42.1%) of the matched 38 patients who received peg-IFN therapy after hepatectomy attained SVR. Among 16 patients who attained SVR, 10 patients received full-dose peg-IFN therapy without dose reduction, whereas 6 patients received a reduced dose of peg-IFN and/or RBV until completion of treatment. Nine patients discontinued peg-IFN therapy because of adverse events such as thrombocytopenia and neutropenia ($n = 2$), skin eruption ($n = 1$), depression ($n = 2$), and severe malaise ($n = 4$). Three patients discontinued peg-IFN therapy because of HCC recurrence. Adherence to peg-IFN therapy was 68.4% in this study. No life-threatening adverse events were observed, and none of the total 15 deaths in both sets of matched patients were related to the IFN treatment or to surgical procedures. The 3- and 5-year overall survival rates of patients ($n = 16$) who attained SVR after peg-IFN therapy were 100% and 100%, respectively; those of patients who did not attain SVR ($n = 22$) were 100 and 85.7%, respectively; and those of patients who did not receive IFN therapy were 76.6 and 50.6%, respectively. There was a statistically significant difference in overall survival among the three groups ($P = 0.005$) (Fig. 2a). However, there was no statistically significant difference in disease-free survival among the three groups ($P = 0.90$) (Fig. 2b).

Table 2 presents the patterns of cancer recurrence and the treatment details of the recurrences in both groups. Twenty-one (55.3%) of the patients who received peg-IFN therapy after hepatic resection and 17 (44.7%) of the patients who did not receive IFN therapy had HCC recurrences after hepatic resection. Regarding the pattern of recurrence, the proportion of patients who had multiple

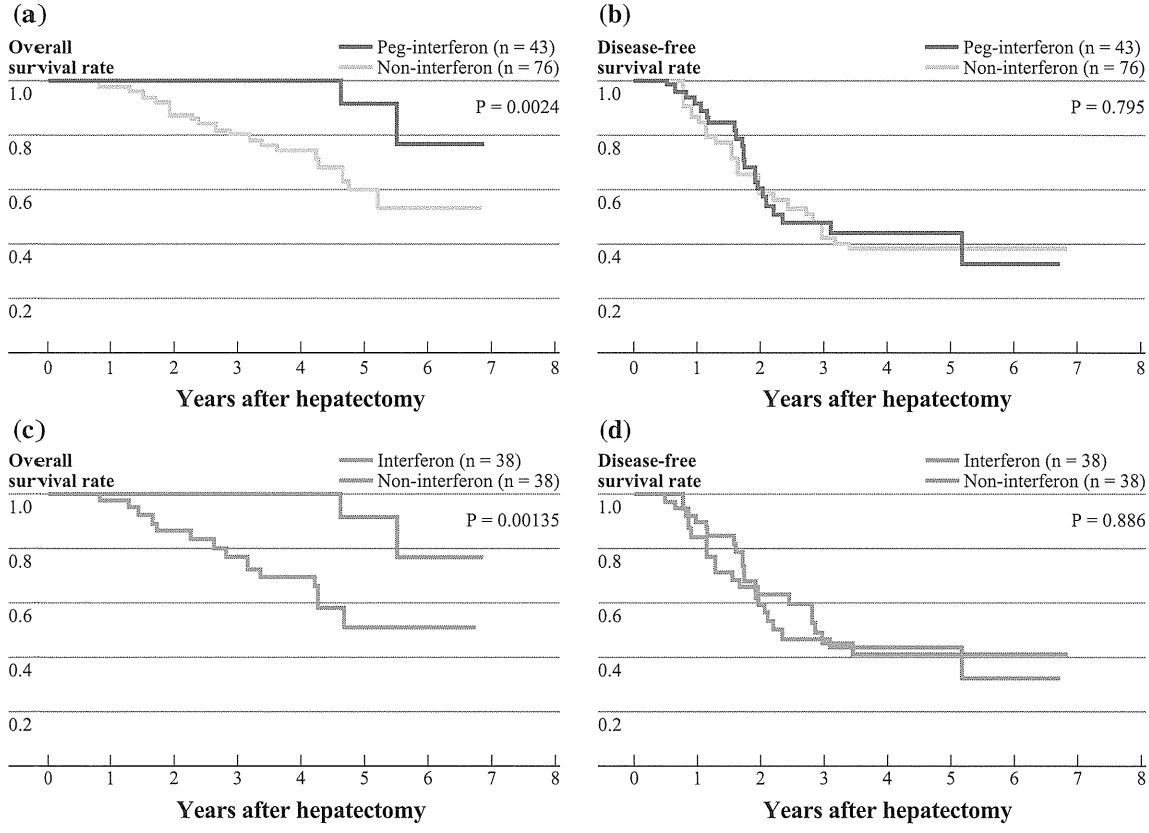

FIG. 1 Overall survival (**a**) and disease-free survival (**b**) of the entire study population of 175 patients with hepatitis C-related HCC with respect to IFN therapy after hepatic resection. Overall survival (**c**) and disease-free (**d**) survival of the matched study population of 76 patients with hepatitis C-related HCC with respect to IFN therapy after hepatic resection

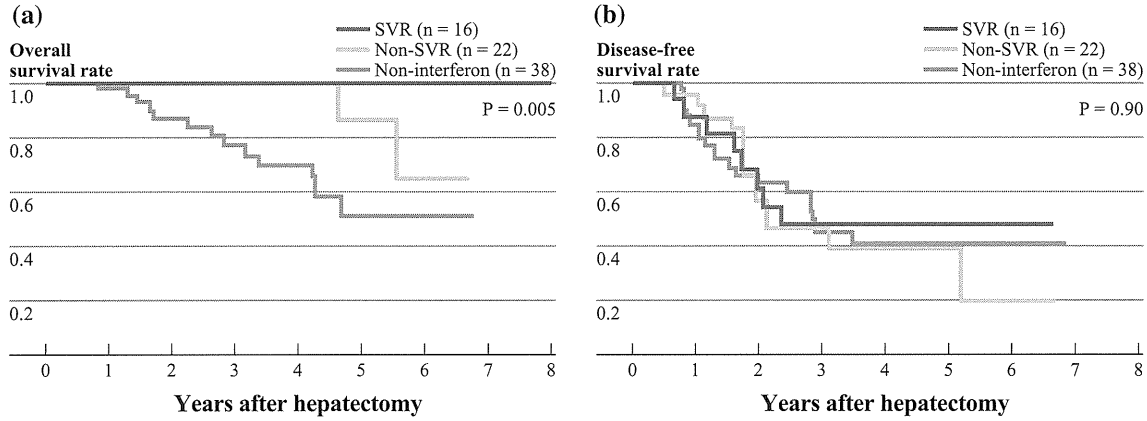

FIG. 2 Overall survival and disease-free survival of patients with hepatitis C-related HCC with respect to SVR after IFN therapy

intrahepatic recurrences (more than four nodules) was significantly lower in the peg-IFN group than in the non-IFN group ($P = 0.0047$). The proportion of patients in whom surgery or RFA was selected for treatment was significantly higher in the peg-IFN group than in the non-IFN group ($P = 0.0346$). Furthermore, regarding re-recurrence of HCC after treatment of the first-recurrent HCC, the 1-year disease-free survival rates of patients after treatment of the first-recurrent HCC was 48.5% in patients ($n = 21$) who received peg-IFN therapy and 12.5% in patients ($n = 17$) who did not receive IFN therapy. There was a statistically significant difference in disease-free survival between the two groups ($P = 0.0012$) (Fig. 3).

A comparison of results of the preoperative liver function test with those of postoperative 1-year liver function tests is presented in Table 3. In patients who received peg-IFN therapy, total bilirubin levels 1 year after surgery were significantly decreased compared with preoperative total bilirubin levels ($P = 0.018$), whereas in patients who did not receive IFN therapy, the total bilirubin level at 1 year after surgery was similar to the total bilirubin level before surgery ($P = 0.107$).

TABLE 2 Recurrence and treatments for recurrence after hepatic resection

	Peg-IFN (+) ($n = 38$)	IFN (−) ($n = 38$)	P value
HCC recurrence[a]: yes	21 (55.3%)	17 (44.7%)	0.359
Pattern of recurrence[b]			0.0047
Intrahepatic (single)	9 (42.9%)	8 (47.1%)	
Intrahepatic (2–3)	10 (47.6%)	1 (5.9%)	
Intrahepatic (multiple)	2 (9.5%)	8 (47.1%)	
Main modalities[b]			0.0346
Repeat hepatectomy	8 (38.1%)	2 (11.8%)	
RFA	8 (38.1%)	4 (23.5%)	
TACE	5 (23.8%)	11 (64.7%)	

peg-IFN pegylated interferon, *RFA* radiofrequency ablation, *TACE* transcatheter arterial chemoembolization

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

[b] Data expressed as number of patients (percentage of patients who had a recurrence)

DISCUSSION

Our results revealed that peg-IFN therapy after hepatic resection improved the outcomes of HCV patients, although the interval of disease-free survival was not prolonged. Peg-IFN therapy after hepatectomy improved hepatic reserve function and suppressed multiple HCC recurrences (more than four nodules). Furthermore, re-recurrence after treatment of first-recurrent HCC after hepatic resection was significantly suppressed in the peg-IFN group compared with that in the non-IFN group. IFN has been reported to exert antitumor effects. IFN increases natural killer cell activity and exhibits antiangiogenic properties.[35,36] IFN has also been reported to be effective in eradicating HCV RNA

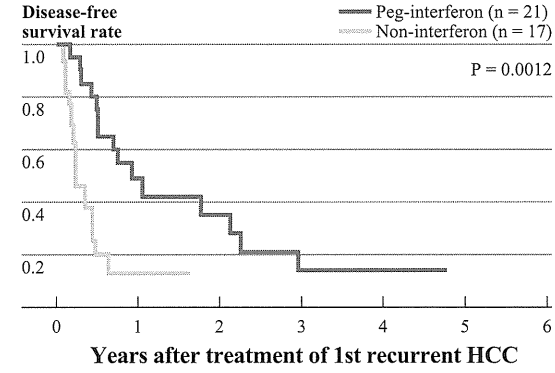

FIG. 3 Comparison of disease-free survival rate after treatment of first-recurrent HCC in patients who received peg-IFN therapy or in those who did not receive IFN therapy

TABLE 3 Comparison of preoperative liver function with 1-year liver function after hepatic resection

	Peg-IFN (+)		*P* value	IFN (−)		*P* value
	Preoperative	1 Year after surgery		Preoperative	1 Year after surgery	
T-Bil (mg/dl)	0.82 ± 0.29	0.71 ± 0.26	0.0189	0.81 ± 0.32	0.92 ± 0.35	0.107
AST (IU/l)	50.1 ± 24.1	45.8 ± 23.5	0.310	42.1 ± 18.9	56.1 ± 26.7	0.0110
ALT (IU/l)	51.3 ± 28.6	36.4 ± 22.8	0.00809	40.3 ± 24.3	49.7 ± 25.8	0.0918
Albumin (g/dl)	3.89 ± 0.80	3.99 ± 0.71	0.251	3.73 ± 0.45	3.75 ± 0.44	0.807

peg-IFN pegylated interferon, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase

from serum and hepatic tissue, thereby preventing deterioration of liver function in patients with HCV infection.[37] IFN prevents worsening of compensated cirrhosis.[18,37] Our results are compatible with those reported in those studies. In the peg-IFN group, most patients with HCC recurrence could undergo curative treatments such as repeat hepatectomy or RFA as a recurrence treatment, because the number of recurrent tumors was usually limited to three. IFN therapy appears to increase survival not only by improving residual liver function and increasing the possibility of radical treatment of recurrences but also by suppressing re-recurrence after the first recurrence of HCC.

The current study also revealed that the overall survival of patients with SVR was significantly better than that of patients without SVR. This result suggests that IFN prolongs the outcomes of patients with HCC after hepatic resection by causing remission of active hepatitis and eradication of HCV RNA in patients who attained SVR after hepatic resection.

In this study, to clarify the impact of peg-IFN therapy on outcomes of HCV-related HCC after hepatic resection, patients who received IFNs such as IFN-α or IFN-β were excluded. RCTs investigating adjuvant effects of IFN after resection or ablation of HCC were performed using IFN-α. Few studies have investigated the effects of peg-IFN plus RBV combination therapy on survival and recurrence after curative resection of HCC. Combination therapy with peg-IFN and RBV has recently been developed, and peg-IFN therapy has resulted in significantly higher SVR rates and better tolerability than treatment with IFN-α.[21,23] In our study, incidence of SVR after hepatic resection was 42.1%, which was higher than that in previous studies that reported an SVR rate of 0–10%.[12–14] The compliance of patients to peg-IFN therapy observed in the present study (68.4%) was higher than that reported elsewhere (approximately 40%).[14] This enhanced efficacy of the peg-IFN formulations might contribute to the prolonged survival of HCC patients after hepatic resection.

In this study, HCC patients who received peg-IFN therapy within 9 months after surgery were enrolled, and HCC patients who experienced recurrence of HCC within 9 months after hepatic resection were excluded from the non-IFN group, because these patients could lose the opportunity to receive IFN therapy for HCC recurrence on being assigned to the peg-IFN therapy group.

Before matching by using the propensity score, the clinical characteristics of the entire study population that can strongly influence outcomes differed significantly between the peg-IFN group and non-IFN group. The proportion of older patients was higher in the non-IFN group than in the peg-IFN group, whereas the proportion of patients who had longer operation times tended to be lower in the non-IFN group than in the peg-IFN group. To overcome bias due to the different distribution of the severity of liver function impairment between the two 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 between the two matched groups. This study had a limitation related to the small sample size after propensity score matching. To overcome this, further examination with larger sample sizes is necessary, and the potential efficacy of peg-IFN therapy must be validated in larger prospective RCTs.

CONCLUSIONS

Several previous RCTs investigating the effects of IFN on survival and tumor recurrence after hepatic resection were inconclusive. However, in the current study, peg-IFN therapy following hepatic resection improved the survival rates of hepatectomized patients with HCV-related HCC. The results of this study suggest that peg-IFN therapy is effective as an adjuvant chemopreventive agent after hepatic resection in patients with HCV-related HCC.

ACKNOWLEDGMENT The authors thank Prof. Junko Tanaka of the Department of Epidemiology, Infectious Disease Control and Prevention, Hiroshima University, for assistance in performing the propensity score analysis.

CONFLICT OF INTEREST The authors have no commercial associations (e.g., consultancies, stock ownership, equity interest, patent/licensing arrangements) that might pose a conflict of interest related to the submitted manuscript.

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Journal of Surgical Oncology 2011;104:3–9

Treatment Strategy for Early Hepatocellular Carcinomas: Comparison of Radiofrequency Ablation With or Without Transcatheter Arterial Chemoembolization and Surgical Resection

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Background: The preferred choice between surgical treatment and radiofrequency ablation (RFA) for the treatment of small resectable hepatocelluar carcinoma (HCC) has become a subject for debate.
Methods: We compared the results of hepatic resection (n = 199) with those of RFA (n = 87), of which 69 patients were treated with transcatheter arterial chemoembolization followed by RFA, for 286 patients with 3 or fewer nodules, none of which exceeded 3 cm in diameter at Hiroshima University Hospital.
Results: In subgroup analysis of single HCC with tumor size exceeding 2 cm in Child-Pugh class A, the disease-free survival time was significantly longer in the surgical resection group than in the RFA group ($P = 0.048$). In the subgroups of a single and multiple HCC with tumor size $\leq$2 cm in Child-Pugh class A, the overall and disease-free survival rates were almost the same for the surgical resection and RFA groups ($P = 0.46$ and 0.58, respectively, in single HCC, and $P = 0.98$ and 0.98, respectively, in multiple HCC).
Conclusion: Surgical resection may provide better long-term disease-free survival than RFA in the subgroup of a single HCC exceeding 2 cm of Child-Pugh class A.
J. Surg. Oncol. 2011;104:3–9.

KEY WORDS: **early hepatocellular carcinoma; hepatectomy; radiofrequency ablation**

INTRODUCTION

Hepatocelluar carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer death worldwide [1]. Although the majority of cases are still found in Asia and Africa, recent studies have shown that the incidence and mortality rates of HCC are increasing in North America and Europe [2]. Over the past two decades, great progress has been made in the diagnosis of HCC using non-invasive diagnostic modalities, and it is feasible to make early detection of HCC. Current options for the treatment of the early HCC consist of surgical resection, liver transplantation, transcatheter arterial chemoemolization (TACE), and percutaneous tumor ablation. These modalities have all been used for HCC patients according to the clinical characteristics of their tumors and the hepatic functional reserve of the patients. Hepatic resection has been shown to be the most efficacious treatment for HCC [3]; however, hepatic resection is limited to patients with good hepatic functional reserve. Radiofrequency ablation (RFA) is a recently introduced technique that is rapidly being adopted worldwide because of its greater efficacy for local cure compared with ethanol injection [4,5]. RFA is usually indicated for patients with three or fewer nodules, none of which exceed 3 cm in diameter [6]. Livraghi et al. [7] showed that RFA is just as effective as surgery for the treatment of very early HCC (single HCC nodules measuring 2.0 cm or less) in terms of sustained local disease control and survival. They advocated that RFA can be considered as the preferred treatment for patients with single HCC of 2.0 cm or less, even when surgical resection is possible. Recent, studies compared local ablation therapies with surgical resection [8–14]. However, few studies have evaluated the results of RFA in comparison with surgical resection within a subgroup (e.g., nodules $\leq$2.0 cm vs. >2.0 cm, and single vs. multiple HCCs) analysis of patients with early HCC (three or fewer nodules that are $\leq$3 cm in diameter). The aim of this retrospective study is to compare the patients with early HCC who were submitted to surgical resection and RFA from these points of view.

PATIENTS AND METHODS

From 2001 to 2007, 286 patients underwent liver resection, or RFA for single or multiple (less than 3) HCC measuring $\leq$3 cm as an initial treatment at Hiroshima University Hospital.

The diagnosis of HCC was based on routine imaging modalities including ultrasonography (US), computed tomography (CT) during hepatic angiography, and magnetic resonance imaging (MRI). HCC was diagnosed based on the following classic imaging manifestations: hyperattenuation on CT during hepatic arteriography and hypoattenuation on CT during arterial portography [15]. In case of hypovascular lesion, fine-needle biopsy was performed to obtain histological confirmation in patients who underwent RFA. Before treatment, all patients underwent liver function tests including bilirubin, albumin,

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Received 30 January 2010; Accepted 11 August 2010
DOI 10.1002/jso.21745
Published online in Wiley Online Library (wileyonlinelibrary.com).

prothrombin time, and indocyanine green retention rate at 15 min (ICGR 15) tests.

Hepatic Resection

In the current study, 199 patients were subjected to surgical resection of early HCC. The surgical procedure was determined according to the extent of the tumor, hepatic reserve function, and the patients' wishes. Liver function was assessed by Child-Pugh classification and ICGR 15. If liver function would allow, anatomic resection (segmentectomy (n = 76), sectionectomy (n = 11), or hemihepatectomy (n = 5)) was performed. In patients with insufficient hepatic reserve, limited resection (n = 107) was performed. For example, right hemihepatectomy could be tolerated if ICGR 15 was in the normal range. One-third of the liver parenchyma could be resected for patients with ICGR 15 of 10–19%, segmentectomy was possible with ICGR 15 of 20–29%, and limited resection was possible with ICGR 15 of 30% and more [16]. The procedures of hepatectomy were the same as those described previously [17,18].

RFA

Eighty-seven patients were subjected to RFA. Patients requesting not to undergo hepatectomy underwent RFA. Among the 87 patients, 69 patients were diagnosed as HCC based on CT imaging. The remaining 18 patients were diagnosed as HCC by histopathological methods. Patients were treated with RFA following TACE, if HCC nodules had hypervascularity. TACE was performed an average 3 days before RFA TACE was performed through the femoral artery using the technique of Seldinger under local anesthesia. An angiographic catheter was inserted selectively into the hepatic feeding artery of a segment or subsegments containing the target tumor. We used cisplatinum (Randa; Nippon Kayaku, Tokyo, Japan) as an anticancer drug mixed with iodized oil (Lipiodol; Nihon Schering, Tokyo, Japan) at a concentration of 10 mg/ml and injected at a dose of 10–40 mg/person. The selected dose was based on tumor size. Injection was discontinued upon full accumulation of iodized oil in the tumor vessels. No gelatin sponge or coil embolization was used after TACE in the present study.

RFA was conducted using a commercially available system (Cool-tip RF system; Radionics, Burlington, MA) and electrode that was 17-gauge. Sixty-nine patients whose tumor had hypervascularity were treated with a combination of TACE with RFA. The remaining 18 patients were treated by RFA alone. All patients underwent RFA with a percutaneous approach under real-time ultrasonographic guidance in a ward setting under local anesthesia and conscious sedation [19]. The treatment response was evaluated using CT image. When the diameter of the non-enhancing area was greater than that of the ablated nodule, RFA was considered to have produced a complete effect. HCCs with incomplete response were reevaluated for a new session.

Follow-up

Follow-up evaluation after the surgery or RFA consisted of blood chemistry tests and measurements of tumor markers including α-fetoprotein (AFP) and Des-γ-carboxy prothrombin (DCP), every month. Patients were examined by ultrasound every 3 months and by computed tomographic (CT) scan every 6 months. When recurrence was indicated by any of these examinations, patients underwent CT during arterial portography and arteriography.

Complications were stratified according to the Clavien classification of postoperative surgical complications [20] and imaging-guided tumor ablation: standardization of terminology and reporting [21]. Major complications were defined as those which required treatment or additional hospitalization, or which resulted in permanent adverse sequelae (Clavien classification grade II or higher). This includes any case in which a blood transfusion or interventional drainage procedure is required.

Treatment for Recurrence

All patients with intrahepatic recurrence were managed with ablative therapies (RFA or ethanol injection), TACE, or surgery including liver transplantation according to the same criteria used at the time of initial resection.

Histopathological Examination

The resected specimens were serially sectioned at 10-mm intervals and examined macroscopically. The criteria used to identify intrahepatic micrometastasis were essentially those proposed by the Liver Cancer Study Group of Japan; that is, tumors surrounding the main tumor with multiple other satellite nodules or small solitary tumors located near the main tumor that are histologically similar or less differentiated than the main tumor [22].

Statistical Analyses

Values for continuous variables are presented as means ± SD. Categorical variables were compared using the chi-square test and continuous variables using Student's *t*-test. Overall survival and disease-free survival analyses were carried out using the Kaplan–Meier methods; comparisons between different groups were carried out using the log rank test. The following variables were examined: age (≥70 vs. <70), sex, positivity for hepatitis C virus (HCV) antibody, ICGR 15 (≥15 vs. <15), Child-Pugh class (A vs. B), main tumor size (>20 mm vs. ≤20 mm), tumor number (single vs. multiple), plasma DCP level (≥100 AU/ml vs. <100 AU/ml), and plasma AFP level (≥100 ng/ml vs. <100 ng/ml). Multivariate analyses for survival and disease-free survival were carried out using the Cox's regression model. The regression model was used to evaluate variables found to be associated with infection by univariate analysis ($P < 0.1$). A *P*-value of less than 0.05 was considered significant. Calculations were performed using SPSS software (version 16; SPSS, Inc., Chicago, IL).

RESULTS

There were no differences in age and gender between the surgical resection and RFA groups. However, the hepatic resection group included more patients with hepatitis B virus (HBV) ($P = 0.049$). With regard to hepatic reserve function, ICGR 15 was significantly better in the surgical resection group than in the RFA group ($P = 0.004$); the ICGR 15 was 19.5 ± 9 in the surgical resection group and 23.7 ± 12 in the RFA group. The surgical resection group included more patients with well-preserved liver function (Child-Pugh class A) without statistical significance ($P = 0.06$). On the other hand, regarding with tumor-related factors, the tumor size and DCP level were significantly greater in the surgical resection group than in the RFA group ($P = 0.001$ and 0.03, respectively), and the tumor number was also greater in the surgical resection group than in the RFA group with statistical significance ($P = 0.023$). The mean follow-up of surgical resection and RFA groups were 35 ± 2.5 and 32 ± 2.5 months, respectively. There was no significant difference in overall survival between two groups ($P = 0.11$); the 3-year overall survival rates were 91% in the surgical resection group and 81% in the RFA group (Fig. 1). There was also no significant difference in disease-free survival between two groups ($P = 0.88$); the 3-year disease-free survival rates were 41% in the surgical resection group and 34% in the RFA group

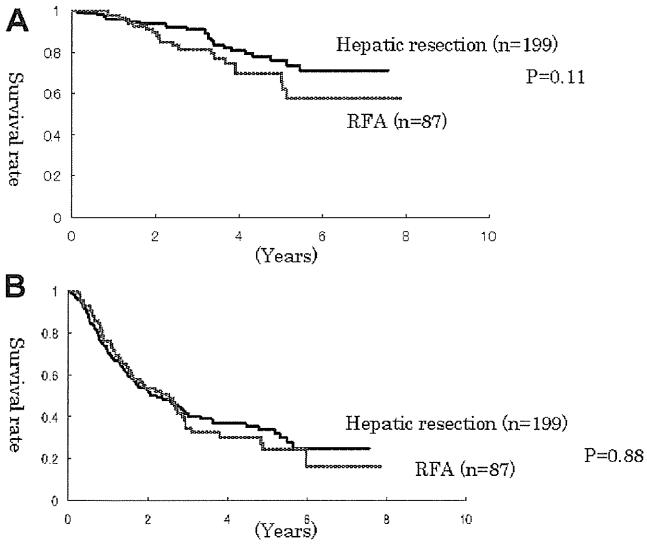

Fig. 1. **A**: Comparison of overall survival rates between patients in the surgical resection and the RFA groups. **B**: Comparison of disease-free survival rates between patients in the surgical resection and RFA groups.

(Fig. 1). Each clinical characteristic including complication was compared between the hepatic resection and RFA groups by univariate analysis, as presented in Table I. There was no mortality during initial hospital stays in both hepatic resection and RFA groups except for one patient who died as a result of suicide within 1 month of hepatic resection. There were no major complications after RFA, whereas major complications occurred in seven patients after hepatectomy. The rate of morbidity after hepatectomy tended to be higher than that after RFA ($P = 0.076$). The hospital stay of hepatectomized patients was significantly longer as compared to that of patients who had underwent RFA ($P = 0.0001$). There was recurrence at the site of the treated tumor in 4 patients who underwent RFA. Complete necrosis was confirmed by imaging in 92% of patients with RFA. Among the four patients who showed recurrence at the site of the treated tumor, three patients had HCC with tumor size exceeding 2 cm (tumor size: 2.0, 2.5, and 3.0 cm), and one patient had HCC with tumor size of 1.5 cm which was located near the liver surface.

Next, subgroup comparisons of overall and disease-free survivals were made between surgical resection and RFA groups (Table II). In subgroup analysis for a single HCC with tumor size exceeding 2 cm in Child-Pugh class A, the disease-free survival was longer in the surgical resection group (n = 72) than in the RFA group (n = 15) with statistical significance ($P = 0.048$); the 3-year disease-free survival rates were 43% in the surgical resection group and 27% in the RFA group (Fig. 2B). In the same subgroup, however, the overall survival was longer in the surgical resection group than in the RFA group without statistical significance ($P = 0.57$); the 3-year overall survival rates were 88% in the surgical resection group and 74% in the RFA group. For multiple HCCs with tumor size exceeding 2 cm in Child-Pugh class A, the overall and disease-free survival rates were longer in the surgical resection (n = 27) than RFA groups (n = 5) without statistical significance. On the other hand, in the subgroup of a single HCC with tumor size $\leq$2 cm in Child-Pugh class A, the overall and disease-free survival rates were almost the same for the surgical resection (n = 53) and RFA (n = 41) groups; the 3-year overall and disease-free survival rates were 95% and 59%, respectively, in the surgical resection group and 94% and 48%, respectively, in the RFA group (Fig. 2A). Moreover, for multiple HCCs with tumor size $\leq$2 cm in Child-Pugh class A, the overall and disease-free survival rates were also almost the same for the surgical resection (n = 30) and RFA groups (n = 11). The subgroup analyses of patients with Child-Pugh class B could not be precisely evaluated due to the small number (less than 4) of cases in each subgroup.

Table III summarizes the results of univariate analyses for all patients according to the clinical characteristics. The Child-Pugh class B ($P = 0.001$) and the tumor number ($P = 0.025$) were significant adverse prognostic factors for overall survival. Similarly, HCV positivity ($P = 0.02$), ICGR 15 $\geq$ 15% ($P = 0.043$), and the tumor number (2 or 3) ($P = 0.0002$) were significant adverse prognostic factors for disease-free survival. In multivariate analyses, Child-Pugh class B ($P = 0.043$) was an independent variable related to adverse overall survival (Table IV). The overall survival rates at 1, 3, and 5 years of 254 patients of Child-Pugh class A were 97%, 91%, and 77%, respectively. The corresponding survival rates of 32 patients of Child-Pugh class B were 94%, 67%, and 56%, respectively.

Table V presents the pathological findings. The incidence of regional cancer spread was significantly lower for HCCs smaller than

TABLE I. Background Characteristics of Patients With Resection or RFA

Variables	Hepatic resection (n = 199)	RFA (n = 87)	*P*-value
Gender (male/female)	137 (68%)/62 (31%)	53 (61%)/34 (39%)	0.19
Age (year)	65.7 ± 9.0	66.3 ± 8.2	0.6
Virus (B/C/others)	38 (19%)/145 (73%)/16 (8%)	9 (10%)/73 (84%)/5 (5%)	0.049
Total bilirubin (mg/dl)	0.86 ± 0.34	0.93 ± 0.36	0.1
Prothrombin time (%)	86.6 ± 14.9	83.7 ± 15.4	0.139
Serum albumin (g/dl)	3.82 ± 0.47	3.78 ± 0.50	0.537
Platelet count (10^4/mm^3)	11.8 ± 7.1	10.5 ± 8.6	0.21
ICG R 15 (%)	19.5 ± 9.0	23.7 ± 12	0.004
Child-Pugh classification (A/B)	182 (91%)/17 (9%)	72 (83%)/15 (17%)	0.06
Tumor size (mm)	2.1 ± 0.63	1.8 ± 0.52	0.001
Tumor number (single/multiple)	132 (66%)/67 (34%)	67 (77%)/20 (23%)	0.023
DCP (AU/ml)	197 ± 756	72 ± 223	0.03
AFP (ng/ml)	310 ± 1322	85 ± 166	0.11
Hospital stay (day)	15 ± 8	8 ± 3	0.0001
Major complications			0.076
Ascites or pleural effusion	2 (1%)	0	
Rupture of esophageal varices	2 (1%)	0	
Biliary leakage	3 (2%)	0	

RFA, radiofrequency ablation; B, hepatitis B virus; C, hepatitis C virus; ICGR 15, indocyanine green retention rate at 15 min; DCP, Des-γ-carboxy prothrombin; AFP, alpha-fetoprotein.

TABLE II. Overall Survival and Disease-Free Survival Rate for Patients of Child-Pugh Class A

	Overall survival rate (%)				Disease-free survival rate (%)			
Factor	1 year	3 years	5 years	*P*-value	1 year	3 years	5 years	*P*-value
Single HCC >2 cm				0.57				0.048
Resection (n = 72)	94	88	83		71	43	36	
RFA (n = 15)	100	74	74		44	27	9	
Multiple HCC >2 cm				0.18				0.98
Resection (n = 27)	96	96	43		60	28	22	
RFA (n = 5)	100	75	38		80	0	0	
Single HCC ≤2 cm				0.46				0.58
Resection (n = 53)	100	95	88		90	59	53	
RFA (n = 41)	97	94	83		81	48	43	
Multiple HCC ≤2 cm				0.98				0.98
Resection (n = 30)	92	92	69		61	22	22	
RFA (n = 11)	100	92	73		80	18	18	

RFA, radiofrequency ablation.

2 cm than for HCC >2 cm; with microvascular invasion (22% vs. 4.3%) and micrometastasis (20.3% vs. 8.3%) being more common in HCCs >2 cm compared to those ≤2 cm.

Ninety-seven (49%) of the patients in the hepatic resection group and 43 (49%) of the patients in the RFA group showed HCC recurrences. The pattern of recurrence and the details of treatments for the recurrences in both groups are shown in Table VI. The main treatment for recurrence was TACE (34%), followed by RFA (28%) and repeat hepatectomy (23%) in the surgical resection group, whereas the main treatment for recurrence was RFA (37%), and followed by TACE (35%) and hepatectomy (16%) in the RFA group. One patient underwent salvage liver transplantation for recurrence in the surgical resection group. The proportion of patients with extrahepatic recurrence tended to be higher in the hepatic resection group ($P = 0.07$), but the modalities of the treatments used for recurrence did not differ among the two groups.

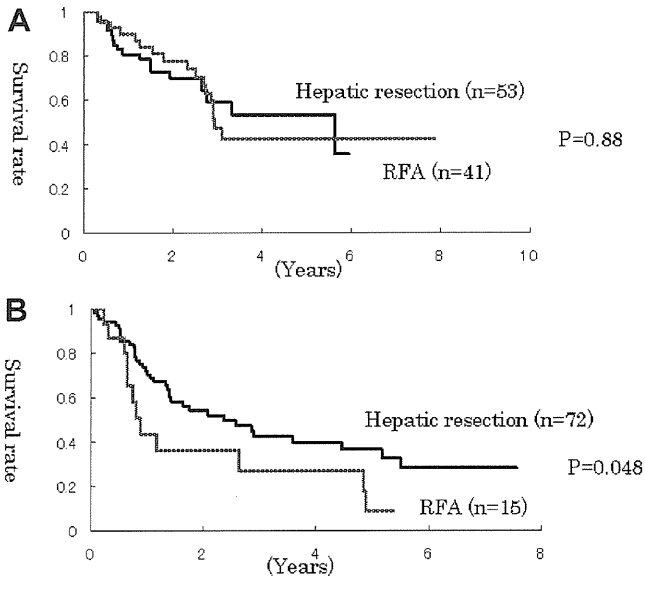

Fig. 2. **A**: Comparison of disease-free survival rates in patients with a single HCC with tumor size less than 2 cm in Child-Pugh A between patients in the surgical resection and RFA groups. **B**: Comparison of disease-free survival rates in patients with a single HCC with tumor size >2 cm in Child-Pugh A between patients in the surgical resection and RFA groups.

DISCUSSION

Livraghi et al. [7] recently demonstrated that the estimated 3- and 5-year survival rates for the potentially operable subgroup (100 patients with a single HCC ≤2 cm) were 89% and 68%, respectively. They showed that the 5-year survival rate in their study was comparable to that reported by the Liver Cancer Study Group of Japan, which revealed a 5-year survival rate of 70% for 2078 patients. They concluded that RFA can be considered the preferred treatment for patients with single HCC ≤2 cm, even when surgical resection is possible, since RFA is much less invasive and has a lower complication rate, and other approaches can be used as salvage therapy for the few cases in which RFA is unsuccessful or unfeasible. In our study, a combination of TACE and RFA was performed in the majority of patients with hypervascular HCC nodules who did not undergo surgical resection. The diagnosis by CT imaging has the possibility of misdiagnosis of HCC for nodule measuring 2.0 cm or less. Vascular occlusion by TACE permits the formation of larger thermal lesions by reducing heat loss [23,24]. In addition, the accumulation of lipiodol might be useful for obtaining the border of the tumors at CT scan after RFA [19]. In our current study, RFA for HCC smaller than 2 cm has overall and disease-free survival rates similar to those for the resection group. The previous histopathologic studies have shown that, although HCC nodules measuring 1.5 cm or less are uniformly well differentiated, those between 1.5 and 2.0 cm in diameter often contain zones of less differentiated tissue with more intense proliferative activity [25]. Takayama et al. [26] also found that among 70 patients with an early single HCC of 2 cm or less in diameter, only 15 HCC lesions were well-differentiated, and microscopic regional spread (vascular invasion and micrometastasis) was identified in 23 (33%) patients. On the other hand, Wakai et al. [27] shown that vascular invasion was more frequent in patients with HCC >2 cm (16/62, 26%) than in patients with HCC ≤2 cm (1/23, 4%, $P = 0.033$). In the current study, the incidence of micrometastasis and microvascular invasion was significantly lower among patients with HCCs 2 cm or less in diameter than among patients with HCCs larger than 2.0 cm in diameter. Recently, Shi et al. [28] found that among patients with HCCs ≤3 cm, 38 (86%) out of 44 identified micrometastases were located within 1 cm of tumor in the same direction of portal venous, and a resection margin of 1.0 cm is recommended for HCCs ≤3 cm. Theoretically, a single electrode insertion can produce a necrotic area of up to 3.0 cm in diameter, thus allowing full ablation of a 2-cm tumor plus a 0.5–1.0 cm safety margin. Our studies have suggested that the choice of RFA does not matter for patients with single or multiple (less than 3) HCC ≤2 cm, if HCC lesions cannot be visualized by US or are close to anatomic structures that might be damaged by RFA.

TABLE III. Univariate Analysis of Clinical Factors for Overall Survival and Disease-Free Survival Rate

	Overall survival rate (%)				Disease-free survival rate (%)			
Factor	1 year	3 years	5 years	*P*-value	1 year	3 years	5 years	*P*-value
Age (year)				0.53				0.7
≤70 (n = 175)	97.6	89.1	76.1		73.1	38.4	31.7	
>70 (n = 111)	94.1	85.5	71.1		72.9	39.4	27.5	
Gender				0.15				0.8
Male (n = 190)	96.6	86.4	72.2		72.4	40.4	34.4	
Female (n = 96)	95.6	89.9	77.3		74.4	35.2	20.2	
Virus				0.09				0.02
HCV (n = 218)	96	86.3	70.9		71.8	31.9	34.4	
Non-HCV (n = 68)	96.8	89.9	84.6		74.4	54	47.6	
Child-Pugh classification				0.001				0.227
A (n = 254)	96.6	90.7	76.6		72.5	39.2	32.6	
B (n = 32)	93.5	67	56.2		74.3	34.3	10.7	
ICGR 15 (%)				0.6				0.043
<15 (n = 148)	96.6	85.1	79		75.1	46.9	36.9	
≥15 (n = 137)	95.6	90.4	68.9		70.2	28.6	22.6	
Tumor size (mm)				0.26				0.07
≤2.0 (n = 157)	96.4	89.8	78.7		79.2	40.2	33.3	
>2.0 (n = 129)	96	85.6	69.8		65.1	36.2	27	
Tumor number				0.025				0.0002
Single (n = 199)	96.8	87.2	80.4		75.9	45.3	36.7	
2 or 3 (n = 87)	95	88.9	59.6		65.1	23.9	16.6	
DCP (AU/ml)				0.11				0.46
<100 (n = 227)	97.1	89.5	76.1		73.1	38.7	30.5	
≥100 (n = 59)	92.8	85.6	69.3		71.2	39.3	30.6	
AFP (ng/ml)				0.65				0.64
<100 (n = 215)	96.5	90.2	74.8		73	38.1	30	
≥100 (n = 71)	95.5	89.4	74.9		73.2	42.1	33.8	
Treatment				0.11				0.88
Resection (n = 199)	95.6	90.9	76		71.4	41.2	33.7	
RFA (n = 87)	97.6	81.4	71		76.5	34.3	24.7	

HCV, hepatitis C virus; ICGR 15, indocyanine green retention rate at 15 min; DCP, Des-γ-carboxy prothrombin; AFP, alpha-fetoprotein.

A preliminary report of the Japanese nationwide survey has shown that surgical resection provides a lower time-to-recurrence rate than RFA does among patients with HCCs no more than three tumors (≤3 cm) [12]. In the current study, we have also shown that in subgroup analysis of a single HCC with tumor size >2 cm in Child-Pugh class A, the disease-free survival was longer in the surgical resection group than in the RFA group with significance. The overall survival was longer in the surgical resection group than in the RFA group, although the result was not significant. Our histopathological study has shown that the incidence of micrometastasis was significantly higher among patients with HCCs exceeding 2 cm in diameter (20%) than among patients with HCCs 2.0 cm or less in diameter (8.3%). These findings have suggested that RFA is less effective than hepatic resection to eradicate venous tumor thrombi and micrometastasis in the adjacent liver in addition to the complete removal of the primary HCC with tumor size >2 cm [29,30]. Surgical resection may provide better long-term disease-free survival than RFA in the subgroup of a single HCC exceeding 2 cm of Child-Pugh class A.

We have shown that in subgroup analysis of multiple HCCs exceeding 2 cm in Child-Pugh class A, the overall survival and the disease-free survival in the surgical resection group was not significant different from that in the RFA group. The strategy for multiple HCCs larger than 2 cm in Child-Pugh class A remains unclear because of small sample number in RFA group.

TABLE IV. Multivariate Analysis of Overall Survival

Variable	HR	95% CI	*P*-value
Child-Pugh class (B vs. A)	1.669	1.016–2.741	0.043

In our study, Child-Pugh class A and a single tumor were significant favorable prognostic factors for overall survival, and HCV negativity, lower ICGR 15, and a single tumor were significant favorable prognostic factors for disease-free survival in univariate analysis, although in a multivariate study only Child-Pugh class A was an independent favorable factor for overall survival. The preliminary report of the Japanese nationwide survey has shown that in multivariate analysis, low tumor marker, tumor size <2 cm, better liver function (Child-Pugh class A), and the presence of HCV infection were favorable factors for overall survival, and a single tumor, low tumor marker levels, small tumor size, the absence of HCV, and younger age were negative factors for recurrence [12]. These results are similar to trends found in the nationwide study.

Radiofrequency is much less invasive, involves a short hospital stay, and has low mortality associated with the procedure. With the intention of avoiding the risk of hepatic failure that can follow hepatic resection, percutaenous ablation treatments have been proposed due to the efficacy, tolerability, and low risk of the procedure. However, in the

TABLE V. Pathological Characteristics of HCC of Patients With Hepatic Resection

Characteristics	HCC >2 cm (n = 103)	HCC ≤2 cm (n = 96)	*P*-value
Histological type			
Well/moderate/poor/unknown	11/75/11/6	22/65/4/5	0.023
Regional cancer spread			
Microvascular invasion	23 (22%)	4 (4.3%)	0.0001
Intrahepatic micrometastasis	21 (20.3%)	8 (8.3%)	0.027

TABLE VI. Recurrence and Treatments for Recurrence After Hepatic Resection or RFA

	Hepatic resection (n = 199)	RFA (n = 87)	*P*-value
HCC recurrence: yes[a]	97 (49%)	43 (49%)	0.84
Pattern of recurrence[b]			0.07
Intrahepatic	86 (89%)	43 (100%)	
Intahepatic + extrahepatic	7 (7%)	0 (0%)	
Extrahepatic	4 (4%)	0 (0%)	
Treatment: yes[b]	93 (96%)	41 (95%)	0.5
Main modalities[b]			0.67
Hepatectomy	23 (24%)	7 (16%)	
RFA	27 (28%)	16 (37%)	
PEI	3 (3%)	2 (5%)	
TACE	33 (34%)	15 (35%)	
Liver transplantation	1 (1%)	0 (0%)	
Others	6 (6%)	1 (2%)	

RFA, radiofrequency ablation; PEI, percutaneous ethanol injection; TACE, transcatheter arterial chemoembolization

[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 who had a recurrence).

current study, hepatic resection has been considered as an acceptable treatment, because the procedure-related mortality was zero after hepatectomy, and there was no significant difference in the incidence of morbidity between the two groups, regardless of the high tendency of the incidence of morbidity after hepatic resection.

Our retrospective study had some drawbacks. Clinical characteristics that can strongly influence outcomes differed significantly between the surgical resection group and the RFA group, as shown in other studies. In the current study, the proportion of the multinodular HCC patients and the levels of DCP were higher in the resection group than those in the RFA group, whereas the proportion of poor function liver reserve was lower in the resection group than that in the RFA group. Because multiple nodules and poor function liver reserve are major risks of recurrence, we conducted subgroup analysis according to the tumor size, tumor number, and Child-Pugh class. Ultimately, a randomized controlled trial would be necessary to prospectively determine if RFA and surgery are comparable therapies for early stage HCC.

In conclusion, RFA can be considered the preferred treatment for patients with single or multiple HCC $\leq$2 cm of Child-Pugh class A. Our results suggest that surgical resection may provide better long-term disease-free survival than RFA does in the subgroup of a single HCC exceeding 2 cm of Child-Pugh class A. A large prospective trial comparing surgical resection with RFA is on-going in the Japanese nationwide study, and thus, clear-cut guidelines are expected to be established in the near future.

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Hindawi Publishing Corporation
Journal of Transplantation
Volume 2011, Article ID 483728, 6 pages
doi:10.1155/2011/483728

Research Article

Evidence for the Immunosuppressive Potential of Calcineurin Inhibitor-Sparing Regimens in Liver Transplant Recipients with Impaired Renal Function

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Received 14 March 2011; Accepted 9 May 2011

Academic Editor: P. Burra

Patients requiring liver transplantation (LT) frequently experience renal insufficiency (RI), which affects their survival. Although calcineurin inhibitor-sparing immunosuppressive regimens (CSRs) are well known to prevent RI, the immune state in recipients receiving CSR remains to be intensively investigated. Among 60 cases of living-donor LT at our institute, 68% of the patients had none to mild RI (non-RI group) and 32% of the patients had moderate to severe RI (RI group). The RI group received a CSR comprising reduced dose of tacrolimus, methylprednisolone, and mycophenolate mofetil, while the non-RI group received a regimen comprising conventional dose of tacrolimus and methylprednisolone. One year after LT, the mean estimated glomerular filtration rate (eGFR) in the RI group had significantly improved, although it was still lower than that of the non-RI group. Serial mixed lymphocyte reaction assays revealed that antidonor T-cell responses were adequately suppressed in both groups. Thus, we provide evidence that CSR leads to improvement of eGFR after LT in patients with RI, while maintaining an appropriate immunosuppressive state.

1. Introduction

Renal insufficiency (RI) has been widely recognized as a serious complication of liver transplantation that significantly compromises patient outcome [1–4]. Since a number of patients already have varying degrees of RI, including hepatorenal syndrome, before undergoing liver transplantation, and since postoperative standard immunosuppression protocols based on calcineurin inhibitors (CNIs) can lead to severe tubular atrophy, interstitial fibrosis, and focal hyalinosis of the small renal arteries and arterioles, a majority of liver recipients develop some degree of RI [5–7]. An analysis of data from the Scientific Registry of Transplant Recipients indicates that the cumulative incidence of stage 4 [estimated glomerular filtration rate (eGFR) < 30 mL/min/1.73 m^2] or stage 5 chronic kidney disease (eGFR < 15 mL/min/1.73 m^2 or need for renal replacement therapy) after liver transplantation is 18% at 5 years [8].

Late renal failure is associated with both pre- and posttransplant factors, including higher concentrations of CNIs both early and late posttransplant and can be predicted by creatinine levels in the first year posttransplant [9, 10]. The recognition of these effects induced interest in strategies using a CNI-sparing immunosuppressive regimen (CSR). Current strategies to overcome CNI toxicity include reduction or withdrawal of CNIs concurrent with switching over to less nephrotoxic drugs like the mammalian target of rapamycin (mTOR) inhibitor or mycophenolate mofetil (MMF) [11–17]. Although these strategies have clearly demonstrated the ability to reduce the incidence of nephrotoxicity in various studies, CSR may result in an increased risk for acute rejection episodes in a subset of patients.

In the present study, we investigated the immune state in liver transplant patients suffering from RI who received a CSR comprising a reduced dose of CNI, methylprednisolone, and MMF. For monitoring the immune-state response to

antidonor allostimulation in these patients, we employed a mixed lymphocyte reaction (MLR) assay using an intracellular carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeling technique. By applying the CFSE-based method, the proliferation of viable $CD4^+$ and $CD8^+$ responder T-cells in response to allostimulation could be separately quantified using multiparameter flow cytometry [18]. The technique allowed us to find that antidonor T-cell responses were adequately suppressed in patients with RI who received the CSR and in patients without RI who received a conventional immunosuppressive regimen.

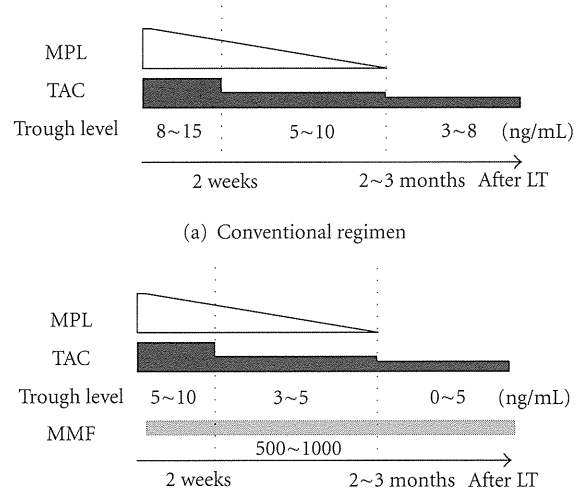
2. Patients and Methods

2.1. Patients. Between January 2003 and December 2009, 122 patients underwent living-donor LTs at Hiroshima University Hospital. Of these, 50 patients infected with hepatitis C virus (HCV) and 12 patients who received liver allografts from ABO-blood group incompatible donors were excluded from the study, because they were treated with the diverse immunosuppressive protocols. For the remaining 60 patients, the relationship between RI prior to LT and the clinical/immunological state after LT was investigated. The following information was collected at the time of the transplant: age, sex, etiology of liver disease, model for end-stage liver disease (MELD) score, and diagnosis of hepatocellular carcinoma (HCC) prior to LT. Renal function was evaluated in each participant by determining eGFR. The eGFR of each participant was calculated from their serum creatinine value (SCr) and their age by using the new Japanese equation [19] as follows:

$$\begin{aligned} &\text{eGFR } (\text{mL/min}/1.73\,\text{m}^2) \\ &\quad = 194 \times \text{Age} - 0.287 \\ &\qquad \times \text{S} - \text{Cr} - 1.094 \ (\text{if female} \times 0.739). \end{aligned} \tag{1}$$

In this study, RI was defined as none to mild (eGFR $\geq$ 60 mL/min/1.73 m^2) and moderate (30–59 mL/min/1.73 m^2) to severe (< 30 mL/min/1.73 m^2). The MELD score was calculated for each patient using the United Network for Organ Sharing (UNOS) formula based on the laboratory values obtained just prior to LT. Patients were monitored for renal function using serum creatinine level and eGFR at 1, 3, 6, and 12 months after LT.

2.2. Immunosuppressive Protocol. The basic immunosuppressive regimen after LT for the non-RI group comprised tacrolimus (TAC) and methylprednisolone, with gradual tapering of doses. Patients with RI received a CSR comprising a reduced dose of TAC, methylprednisolone, and MMF (Figure 1). In the conventional regimen, the trough whole blood levels of TAC were maintained between 8 and 15 ng/mL in the first few postoperative weeks and between 5 and 10 ng/mL thereafter. In the CSR, the trough whole blood levels of TAC were maintained between 5 and 10 ng/mL in the first few postoperative weeks and between 3 and 5 ng/mL thereafter.



(a) Conventional regimen

(b) CNI-sparing immunosuppressive regimen

FIGURE 1: Immunosuppressive protocol after liver transplantation. The basic immunosuppressive regimen comprised tacrolimus (TAC) and methylprednisolone (MPL), with doses gradually being tapered off. The trough whole blood levels of TAC were maintained between 8 and 15 ng/mL in the first few postoperative weeks and between 5 and 10 ng/mL thereafter (a). Renal insufficiency (RI) group received CNI-sparing immunosuppressive regimen (CSR) consisting of TAC reduction and concomitant use of mycophenolat mofetil (MMF) (b).

2.3. Immune Monitoring by an In Vitro MLR Assay. For monitoring the immune state, an in vitro MLR assay was performed at 1, 3, 6, and 12 months after LT. Briefly, peripheral blood mononuclear cells prepared from the blood of the recipients, donors, and healthy volunteers with the same blood type as the donors (third-party control) for use as the stimulator cells were irradiated with 30 Gy, and those obtained from the recipients for use as responder cells were labeled with 5 lm CFSE (Molecular Probes Inc., Eugene, OR, USA), as described previously [18]. The stimulator and responder cells were incubated for 5 days. CFSE stably stains intracellular proteins without causing toxicity, and the fluorescence intensity of each stained cell segregates equally among daughter cells during cell division, resulting in sequential halving of the cellular fluorescence intensity with every successive generation. After culturing for MLR, the harvested cells were stained with either phycoerythrin- (PE-) conjugated antihuman CD4 or PE-conjugated antihuman CD8 monoclonal antibodies and subjected to analysis by flow cytometry. All analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). T-cell proliferation was visualized by the serial-halving of the fluorescence intensity of CFSE. $CD4^+$ and $CD8^+$ T-cell proliferation and stimulation index were quantified using a method described previously [18].

2.4. Statistical Analysis. Quantitative variables were expressed as mean ± standard deviation (SD) or median (range). Categorical variables were presented as values and

Table 1: Patient characteristics at living donor liver transplantation.

(eGFR (mL/min/1.73 m^2))	Non-RI group (n = 41) (94.8 ± 26.9)	RI group (n = 19) (42.5 ± 15.9)	P value
Age at LT (years)	49.2 ± 11.5	52.9 ± 9.0	0.23
Male sex—n (%)	21 (51.2)	13 (68.4)	0.21
Primary diagnosis—n (%)			0.63
HBV	15 (36.6)	9 (47.4)	
Alcoholic	8 (19.5)	5 (26.3)	
AIH	4 (9.8)	1 (5.3)	
Others	14 (34.1)	4 (21.1)	
MELD	16.5 ± 7.1	24.7 ± 10.7	< 0.01
eGFR at 1st year after LT (mL/min/1.73 m^2)	77.2 ± 28.2	60.1 ± 13.5	< 0.01
eGFR > 60 at 1st year after LT—n (%)	26 (72.2)	10 (58.8)	0.33
AR within 1st year—n (%)	10 (24.4)	5 (26.3)	0.87
Bacterial infections—n (%)	13 (31.7)	8 (42.1)	0.43
Fungal infections—n (%)	4 (9.8)	4 (21.1)	0.23
CMV infections—n (%)	10 (24.4)	7 (36.8)	0.32

RI, renal insufficiency; LT, liver transplantation; HBV, hepatitis B virus; AIH, Autoimmune hepatitis; eGFR, estimated glomerular filtration rate; MELD, model for end-stage liver disease; AR, acute rejection; CMV, cytomegalovirus. Data are expressed as means ± standard deviation. Difference with $P < 0.05$ was considered significant.

percentages. Student's t-test, Mann-Whitney test, chi-square test, and Fischer's exact test were used to compare variables between the two groups. Paired t-tests were performed to compare continuous variables throughout the study period. The Kaplan-Meier analyses were used to compare time-to-event variables. P Values < 0.05 were considered statistically significant.

3. Results

The 60 patients included 34 males and 26 females; their ages ranged from 20 to 69 (median 52) years. The primary diseases in these patients included hepatitis B virus-related cirrhosis in 24 patients (of these, 18 patients had HCC), alcoholic cirrhosis in 13 patients (of these, 6 patients had HCC), autoimmune hepatitis in 5 patients (of these, 1 patient had HCC), and other diseases in 18 patients.

Before the LTs, 68% of the patients had none to mild RI (non-RI group; mean eGFR, 94.8 ± 26.9 mL/min/1.73 m^2) and 32% of the patients had moderate to severe RI (RI group; mean eGFR, 42.5 ± 15.9 mL/min/1.73 m^2). The characteristics of these patients are listed in Table 1. There was a difference in MELD score between the groups. Mean TAC trough levels during the first year after LT in the non-RI and RI groups are shown in Figure 2(a). There were differences in mean TAC trough levels during 3 months after LT between the groups. One year after the LDLTs, the mean eGFR in the non-RI group had significantly deteriorated (from 94.8 ± 26.9 to 77.2 ± 28.2 mL/min/1.73 m^2, $P < 0.01$). In contrast, the mean eGFR in the RI group had significantly improved after LT (from 42.5 ± 15.9 to 60.1 ± 13.5 mL/min/1.73 m^2, $P < 0.01$), although it was still lower than that of the non-RI group (Figure 2(b)). Notably, 53% of the patients in the RI group were completely cured of RI by 1 year after LT. None of the patients had severe RI at 1 year after LT nor required chronic hemodialysis during the observation period.

To evaluate the immune status of these patients, we employed a serial MLR assay using a CFSE-labeling technique. Lack of proliferation of both $CD4^+$ and $CD8^+$ T-cells in the antidonor CFSE-MLR assay indicates suppression of the antidonor response, whereas a remarkable proliferation of these T-cells reflects a strong antidonor response. In both groups, limited $CD4^+$ and $CD8^+$ T-cell proliferation was observed in the antidonor responses as compared with the anti-third-party responses through the first year. At 1 month after LT, the average of stimulation index (SI) for $CD4^+$ T-cells in response to anti-third-party stimulation was >2 (the average value in healthy volunteers without any immunosuppressive treatment) that is, there was a normal response in the anti-third-party (Figures 3(a) and 3(b)). At 1 year after LT, the average of SIs for $CD4^+$ and $CD8^+$ T-cells in response to both antidonor and anti-third-party stimulation was <2 (Figures 3(c) and 3(d)). There were no significant differences in acute rejection rates, bacterial, fungal, or cytomegalovirus infection rates and patient survival between the groups (Table 1).

4. Discussion

Chronic RI is a serious complication in liver transplantation that significantly compromises patient survival and outcome. Depending on the criteria applied for a definition of chronic renal insufficiency and the duration of followup, the reported rate of chronic renal insufficiency after liver transplantation may vary from 10% to 80% [1, 20–22]. CNI toxicity has been defined as one of the possible risk factors for renal insufficiency in long-term liver transplant survivors. It has been shown that exposure to CNIs within the first 6 months

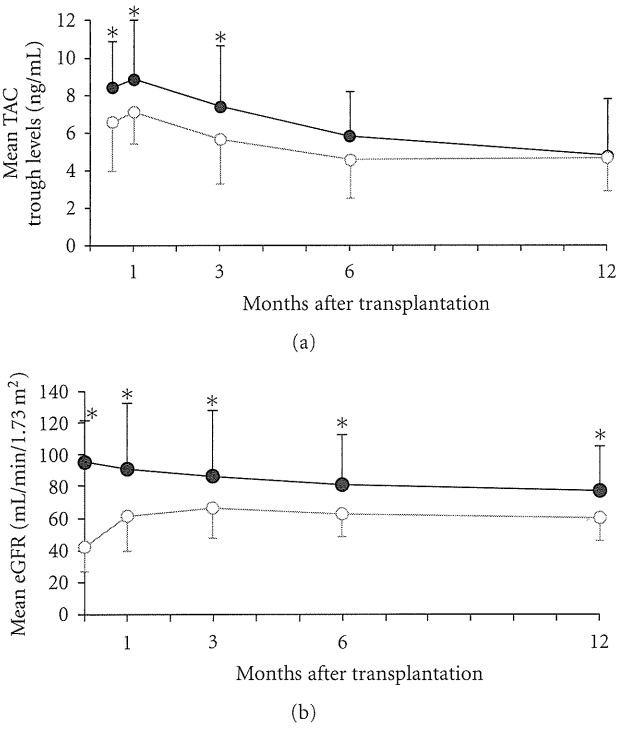


FIGURE 2: Kinetics of mean trough levels of tacrolimus and mean estimated glomerular filtration rate (eGFR) in the RI group and non-RI group during the first year after transplantation. (a) Mean trough levels of tacrolimus in the non-RI group (black line) and RI group (gray line). (b) Mean estimated glomerular filtration rate (eGFR) in the non-RI group (black line) and RI group (gray line). Data are median ± SD of values. $^*P < 0.05$.

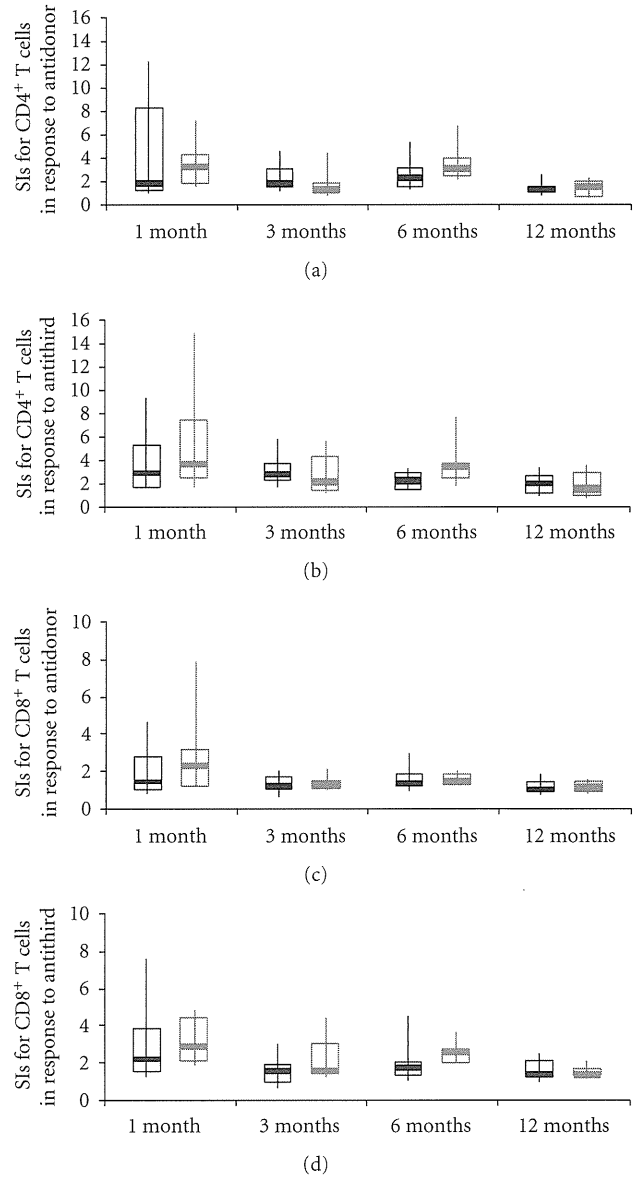


FIGURE 3: Kinetics of stimulation index in the RI group and non-RI group during the first year after transplantation. Stimulation index (SI) of each of the CD4⁺ T-cell (a, b) and CD8⁺ T-cell (c, d) subsets in the antidonor (a, c) and anti-third-party (b, d) MLR in patients in non-RI group (black line) and RI group (gray line). CD4⁺ and CD8⁺ T-cell proliferation and their SIs were quantified as follows. The number of division precursors was extrapolated from the number of daughter cells of each division, and the number of mitotic events in each of the CD4⁺ and CD8⁺ T-cell subsets was calculated. Using these values, the mitotic index was calculated by dividing the total number of mitotic events by the total number of precursors. The SIs of allogeneic combinations were calculated by dividing the mitotic index of a particular allogeneic combination by that of the self-control. The box plot represents the 25th to 75th percentile, the dark line is the median, and the extended bars represent the 10th to the 90th percentile.

after liver transplantation represents a risk factor for renal failure [23]. The GFR at 1 year had a better correlation with later renal function than the pretransplant GFR [24]. The recognition of these facts induced interest in preventing CNI toxicity. It has also reported that the use of adjunctive MMF immediately after LT might protect against CNI nephrotoxicity, potentially without the need for dose reduction or increased risk of adverse events [25]. Therefore, current strategies to overcome CNI toxicity include reduction or withdrawal of CNIs along with switching to mTOR inhibitor or MMF-based regimens [11, 12, 14, 15, 26–28]. These strategies have been documented in several recent and ongoing trials to achieve an improvement in renal function in a large proportion of liver transplant patients.

In our CSR using MMF, wherein our study results agree with the results from previous studies, patients with pre-transplant renal insufficiency were associated with less impairment of renal function without an increased frequency of rejection, infection, or patient survival. In addition to this clinical evidence for the usefulness of the CSR using MMF, the present study provides immunological evidence, by analyzing the data obtained from an MLR assay, that antidonor T-cell responses were adequately suppressed in patients who received the CSR and in patients who received the conventional immunosuppressive regimen. Notably, the individual variations of SIs of CD4⁺ T-cell and CD8⁺ T-cell subsets on antidonor T-cell responses in patients who received the CSR were smaller than those in patients who

received the conventional regimen, although the average values of both were similar. This might be explained by the possibility that the CSR comprising triple immunosuppressive drugs was equally effective in a wide variety of patients.

Several limitations of this study are present. Our sample size was relatively small without long-term followup, and single-center retrospective data are reported. Since the 2 groups of patients are not perfectly comparable as renal impairment can reduce immune responses, we could not rule out a possibility that reduced CNI, without necessarily adding MMF, may be sufficient for the treatment of these patients.

We excluded HCV positive cases and ABO-blood group incompatible cases from the study because of diverse protocol (In brief, in patients with HCV infection, methylprednisolone is not administered, which may be beneficial for preventing enhanced viral replication. Instead, basiliximab and MMF are usually administered to such patients. In ABO-blood group incompatible cases, anti-CD20 monoclonal antibody is administered for eliminating temporarily B cells 2 weeks before transplantation, and simultaneously commencing administration of CNI and MMF.). Hence, the effect of CSR in RI patients with those backgrounds remains to be elucidated. Nevertheless, this first evaluation of the immune state in liver transplant patients suffering from RI received a CSR was essential before to propose an evaluation at a larger scale.

In conclusion, patients with pre-transplant RI receiving CSR under immunological monitoring using an MLR assay were associated with less impairment of renal function without an increased frequency of rejection or patient survival. Antidonor T-cell responses were adequately suppressed in these patients as well as in patients who received the conventional immunosuppressive regimen comprising a standard dose of CNI.

Abbreviations

AIH: Autoimmune hepatitis
AR: acute rejection
CFSE: carboxyfluorescein diacetate succinimidyl ester
CMV: cytomegalovirus
CNI: calcineurin inhibitor
CSR: CNI sparing immunosuppressive regimen
eGFR: estimated glomerular filtration rate
HBV: hepatitis B virus
HCV: hepatitis C virus
LT: liver transplantation
MELD: model for end-stage liver disease
MLR: mixed lymphocyte reaction
MMF: mycophenolate mofetil
mTOR: mammalian target of rapamycin
MPL: methylprednisolone
RI: renal insufficiency
SI: stimulation index
TAC: tacrolimus.

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Research Article

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Rho inhibitor prevents ischemia–reperfusion injury in rat steatotic liver

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Background & Aims: Hepatic stellate cells are thought to play a role in modulating intrahepatic vascular resistance based on their capacity to contract via Rho signaling. We investigated the effect of a Rho-kinase inhibitor on ischemia–reperfusion injury in the steatotic liver.

Methods: Steatotic livers, induced by a choline-deficient diet in rats, were subjected to ischemia–reperfusion injury. Hepatic stellate cells isolated from steatotic livers were analyzed for contractility and Rho signaling activity. The portal pressure of the perfused rat liver and the survival rate after ischemia–reperfusion were also investigated.

Results: Hepatic stellate cells from steatotic livers showed increased contractility and upregulation of Rho-kinase 2 compared with those from normal livers. Furthermore, endothelin-1 significantly enhanced the contractility and phosphorylation level of myosin light chain and cofilin in hepatic stellate cells isolated from steatotic livers. A specific Rho-kinase inhibitor, fasudil, significantly suppressed the contractility and decreased the phosphorylation levels of myosin light chain and cofilin. Serum levels of endothelin-1 were markedly increased after IR in rats with steatotic livers, whereas fasudil significantly decreased endothelin-1 serum levels. Rats with steatotic livers showed a significant increase in portal perfusion pressure after ischemia–reperfusion and a significant decrease in survival rate; fasudil treatment significantly reduced these effects.

Conclusions: Activation of Rho/Rho-kinase signaling in hepatic stellate cells isolated from steatotic livers is associated with an increased susceptibility to ischemia–reperfusion injury. A Rho-kinase inhibitor attenuated the activation of hepatic stellate cells isolated from steatotic livers and improved ischemia–reperfusion injury in steatotic rats.

Introduction

Liver steatosis increases the risk of postoperative morbidity and mortality after liver surgery including liver transplantation [1–3]. Ischemia–reperfusion (IR) injury is one of the most critical complications commonly associated with liver surgery [4–6]. Although it is known that steatotic liver (SL) is particularly vulnerable to IR injury, the mechanisms underlying this increased susceptibility have not yet been clarified.

Experimental studies have indicated that the degree of steatosis is correlated with hepatic microcirculatory disturbances [4,5]. Fat droplet accumulation in the cytoplasm of hepatocytes is associated with an increase in cell volume, which may result in the partial or complete obstruction of the hepatic sinusoidal space and the reduction of sinusoidal blood flow. A continuous state of chronic cellular hypoxia persists in fatty hepatocytes, predisposing the SL to IR injury [7]. The sinusoidal lumens are narrowed by fibrin microthrombi and cellular debris during reperfusion, further decreasing sinusoidal perfusion.

Hepatic stellate cells (HSCs) play an important role in the regulation of hepatic microcirculation. HSCs undergo contraction or relaxation in response to certain stimuli and, as a result, regulate microcirculation by increasing or decreasing the diameter of the sinusoidal lumen [8]. HSCs also play an important role in IR injury [9]. Because HSCs are oxygen-sensing cells [10], they are likely to be activated by exposure to IR-induced oxidative stress, resulting in the disruption of hepatic microcirculation.

The Rho family of small GTPases is known to regulate cell shape and motility through reorganization of the actin cytoskeleton [11]. One of the putative Rho target proteins, the serine/threonine kinase ROCK, mediates cytoskeleton-dependent cell functions by enhancing the phosphorylation of myosin light

Keywords: Steatosis; Ischemia–reperfusion injury; Hepatic stellate cell; Rho-kinase; Fasudil.

Received 19 November 2010; received in revised form 7 April 2011; accepted 29 April 2011; available online 12 July 2011

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Abbreviations: IR, ischemia–reperfusion; SL, steatotic liver; HSCs, hepatic stellate cells; ROCK, Rho-kinase; MLC, myosin light chain; P-MLC, phosphorylated myosin light chain; NL, normal liver; fasudil, fasudil hydrochloride hydrate; NO, nitric oxide; L-NAME, *N*-nitro-L-arginine methyl ester; ET-1, endothelin-1; P-Cofilin, phosphorylated cofilin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; H&E, hematoxylin and eosin; TUNEL, TdT-mediated dUTP-digoxigenin nick-end labeling; HSCs-SL, hepatic stellate cells isolated from rat steatotic liver; HSCs-NL, hepatic stellate cells isolated from rat normal liver; SECs, sinusoidal endothelial cells.

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Journal of Hepatology **2012** vol. 56 | 146–152

chain (MLC) [12]. An increase in phosphorylated MLC (P-MLC) increases the contractility of actomyosin and causes smooth muscle contraction [13]. In addition, P-MLC facilitates the clustering of integrins and the bundling of actin fibers [14], resulting in stimulus-induced cell adhesion and motility.

The contraction of HSCs narrows the sinusoidal lumen and reduces hepatic microcirculatory flow via Rho signaling. We reported previously that the Rho/ROCK signaling pathway played an important role in the activation of HSCs and that a ROCK inhibitor attenuated hepatic injury after warm IR and orthotopic liver transplantation in a rat model [9]. Recently, HSC activation has been shown to be correlated with the severity of steatosis in the liver [15–17]. However, little is known about the connection between activated HSCs and IR injury in SL. Furthermore, there are few studies on the involvement of Rho signaling in the activation of HSCs in SL.

The aim of the present study was to investigate the association between Rho signaling and the activation of HSCs in SL. We also examined whether inhibition of the Rho/ROCK pathway could ameliorate IR injury in the steatotic rat liver.

Materials and methods

Animals

Four-week-old male Wistar rats were purchased from Charles River Breeding Laboratories (Osaka, Japan). Rats were fed either a choline-deficient diet (Hiroshima Institute for Experimental Animals, Hiroshima, Japan) to encourage the development of SL, or a normal diet, which resulted in the development of a normal liver (NL). All animal experiments were performed according to the guidelines set by the US National Institutes of Health (1996).

Liver IR

Under anesthesia, whole rat livers were subjected to warm ischemia by clamping the hepatic artery and portal vein with microvascular clips. The specific ROCK inhibitor fasudil hydrochloride hydrate (fasudil; kindly donated by Asahi Kasei Co., Tokyo, Japan) was used to investigate the effect of ROCK inhibition on liver IR injury. Selected rats were pretreated with 10 mg/kg fasudil (intraperitoneal injection) 30 min before the induction of ischemia.

Isolation of HSCs

HSCs were isolated from rat livers according to previously described procedures [9,18]. Purity was estimated by ordinal light and fluorescence microscopic examination and by indirect enzyme immunoreactivity with an antidesmin antibody (Dako, Versailles, France). HSCs were grown in standard tissue culture plastic flasks in Dulbecco's minimum essential medium with 10% fetal bovine serum and antibiotics.

Collagen gel contraction assay

The contractility of the HSCs was evaluated using hydrated collagen gel lattices on 24-well culture plates as described previously with some modifications [9,19]. To investigate the influence of nitric oxide (NO) on HSCs, the NO synthase inhibitor *N*-nitro-L-arginine methyl ester (L-NAME; Cayman Chemical, Ann Arbor, MI) was used.

Western blot analysis

Primary rat HSCs were left untreated or were treated with 10 μM fasudil and/or 5 nM endothelin-1 (ET-1; Sigma–Aldrich Inc., Tokyo, Japan) for 30 min before homogenization in lysis buffer (Cell Lysis Buffer; Cell Signaling Technology, Danvers, MA). Western blot analysis was performed according to previously described procedures with some modifications [20]. Specific antibodies against β-actin were from Abcam (Tokyo, Japan); those against MLC were from Sigma–Aldrich Inc., and those against P-MLC, cofilin, phosphorylated cofilin (P-Cofilin), and Rho-kinase 2(ROCK2) were from Cell Signaling Technology. The protein expression of ROCK2 was normalized to the level of β-actin. The phosphorylation levels were normalized to the levels of total MLC or cofilin protein expression.

Biochemical assessment

Blood samples were collected from the inferior vena cava. Serum ET-1 concentrations were measured using an Endothelin-1 EIA kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA) according to the manufacturer's instructions. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were assayed by standard enzymatic methods.

Measurement of portal perfusion pressure in the isolated rat liver

Portal pressure in isolated perfused rat livers was measured according to previously described procedures, with some modifications [21]. The perfusion with Krebs–Henseleit buffer (Sigma–Aldrich Inc.) was continued until the monitored inlet pressure value became stable at a constant flow rate of 0.3 ml min^{-1} liver $volume^{-1}$ (ml).

Confocal immunofluorescence and histological study

Phalloidin staining of isolated HSCs and liver sections was performed according to previously described procedures [22]. Samples were observed under a conventional fluorescence microscope or a laser confocal microscope. For the histological study, liver specimens were collected from the middle hepatic lobe after IR. Formalin-fixed liver tissue sections were stained with hematoxylin and eosin (H&E) and examined microscopically. To assess the activity of HSCs in liver sections, phalloidin staining was performed. To assess the grade of the steatosis, sections were stained for oil red O. Furthermore, the detection of apoptosis in liver tissue sections was achieved by TdT-mediated dUTP-digoxigenin nick-end labeling (TUNEL) staining as previously reported [23].

Statistical analysis

Survival rates were compared using the Kaplan–Meier method and analyzed by the log-rank test. Other data are expressed as average values (SD). Statistical analysis among experimental groups was performed using the *t*-test. *p* values less than 0.05 were considered statistically significant. Statistical analyses were performed using SPSS software, version 16 (SPSS Japan Inc., Tokyo, Japan).

Results

Changes in the morphology of HSCs isolated from steatotic rat livers

At 10 weeks, rats fed a choline-deficient diet developed liver steatosis, characterized by more than 60% of fatty filtration in the hepatocytes with few inflammatory cells and slight fibrosis (Fig. 1A). HSCs isolated from rat SLs (HSCs-SL) showed increased stress fiber formation and F-actin expression compared to HSCs isolated from normal rat livers (HSCs-NL), which were suppressed by fasudil treatment (Fig. 1B). Phalloidin staining of liver sections showed stress fiber formation and F-actin expression around sinusoidal spaces in SL after IR, as well as the suppression of these changes by fasudil (Supplementary Fig. 1).

Contractility of HSCs isolated from normal and steatotic rat livers

To evaluate differences in contractility, HSCs-NL and HSCs-SL were cultured on hydrated collagen gels. Contraction was measured as the reduction in the initial area of the gel. In the absence of vasoactive agents, the areas of the gels with HSCs-SL were