(total, 44 HCCs) would be needed to detect a significant difference in the number of CT scans with 5% type I error and 80% power using Student's t test. This calculation was performed prior to initiation of the study. Values of $p < 0.05$ were considered to represent a statistically significant difference. SPSS version 15.0 software (SPSS, Chicago, IL) was used for all analyses.

RESULTS

The clinical characteristics of patients (age, sex, TNM stage, tumor size, localization, Child-Pugh status) did not differ significantly between the 2 groups (Table 1). Mean number of CT scans was 1.6 ± 0.7 (range, 1–4) in Group 1 and 1.1 ± 0.2 (range, 1–2) in Group 2 ($p < 0.01$). The mean number of RFA sessions was 1.6 ± 0.7 (range, 1–4) in Group 1 and 1.8 ± 0.9 (range, 1–4) in Group 2 with no significant differences between the 2 groups. Local recurrence rate at 1 year occurred in 1 of 25 cases (4.0%) in Group 1 and 2 of 25 nodules (8.0%) in Group 2. No major complication occurred after RFA in either group. Minor complications (predominantly elevated temperature) were observed in about 50% of patients (16 patients in Group 1; 17 patients in Group 2). No patient showed reduced liver function, including changes in prothrombin time, cholinesterase, albumin, or total protein. Two of 25 nodules in Group 2 were evaluated as showing sufficient safety margin by VUS-CEUS but were evaluated as having insufficient safety margin by CT.

TABLE 1
Clinical Characteristics of Patients in the CT Group (Group 1) and VUS-CEUS Group (Group 2)

	Group 1 (N = 25)	Group 2 (N = 25)	p value
Sex (M:F)	22:3	16:9	NS
Age (yr)	66.1 ± 7.1	67.0 ± 8.9	NS
TNM stage (1:2:3)	6:12:7	5:10:10	NS
Tumor localization (S1: left: middle: right anterior: right posterior)	1:4:4:9:7	0:6:3:9:7	NS
Tumor size (mm)	17.2 ± 8.9	17.0 ± 6.5	NS
Child-Pugh-classification (A:B)	21:4	19:6	NS

Abbreviation: NS, not significant.

DISCUSSION

Determining whether the tumor was ablated completely and whether sufficient ablated margins were achieved is critical for local control of HCC.[20,25–29] CEUS has recently been reported as a useful procedure to assess the efficacy of RFA of HCC.[20] Evaluation of the vascularity of residual tumors with CEUS is comparable to CT, and CEUS can be used in patients allergic to iodine. Patients with renal failure may also be good candidates for CEUS. Moreover, this approach is less costly and does not involve any exposure to radiation.

On CEUS the ablated area is visualized as a homogeneous nonenhanced area in the postvascular phase (Figure 1B), and identifying the exact margin of the ablated volume is difficult due to the inability to distinguish between ablated tumor and ablated parenchymal tissue.[20] We thus used VUS imaging reconstructed from 3D CT volume data. This allows assessment of the ablated margin by comparing between VUS images depicting location of the tumor before RFA and CEUS images depicting the ablated area after RFA. We have already reported that compared with the gold standard of CT, this method had a sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy of 92.3%, 76.9%, 80%, 90.9% and 84.6%, respectively.[23] As a result, the number of CT scans required after RFA can be reduced.[23]

The present randomized control trial compared the number of CT scans required to assess the efficacy of RFA between a group using CT alone and a group using VUS-CEUS with CT. In this study, 2 lesions whose RFA was evaluated by VUS-CEUS (Group 2) were considered as completely ablated, but the ablated area was actually insufficient according to CT. Those 2 nodules were located where no obvious anatomical landmarks were present, so a pair of identical VUS and CEUS scans could not be generated and ablated tumor tissue could not be unequivocally identified. Success of RFA of other nodules could be assessed by VUS-CEUS. Rates of HCC recurrence did not differ significantly between Groups 1 and 2. This means that evaluating the efficacy of RFA using VUS-CEUS allows accurate determination of ablated margins.

In this study, to avoid disadvantaging Group 2 patients, all HCC nodules in Group 2 treated successfully according to VUS-CEUS were also checked by CT before discharge from hospital. Indeed, recurrence and complication rates did not differ significantly between the 2 groups. Assessing the success of RFA by VUS-CEUS is a promising method and the number of CT scans was significantly decreased using VUS-CEUS. This may contribute to reducing the costs, X-ray

exposure, and occasional risk associated with contrast agents for CT.

When this study was started, the contrast agent SH U 508A (Levovist) was the only agent available for use in Japan. However, perflubutane gas is now available as a new contrast agent (Sonazoid; Daiichi-Sankyo, Tokyo, Japan). Perflubutane gas provides a parenchyma-specific contrast image based on accumulation in Kupffer cells. The efficacy of using this new contrast agent should be confirmed using the same protocol.

In conclusion, use of VUS-CEUS could decrease the number of CT scans required to assess success of RFA of HCC, without increasing the risk of local recurrence or decreased liver function.

REFERENCES

1. El Serag HB, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. N Engl J Med 1999;340:745.
2. Taylor Robinson SD, Foster GR, Arora S, et al. Increase in primary liver cancer in the UK, 1989–94. Lancet 1997;350:1142.
3. Okuda K, Okuda H. Primary liver carcinoma. In: McIntyre N, Benhamou T, Bircher J, editors. Oxford text book of clinical hepatology. Volume 2. Oxford, England: Oxford University Press; 1991, p 1019.
4. Bruix J, Sherman M, Llovert JM, et al. EASL Panel of Experts on HCC. Clinical management of hepatocellular carcinoma. Conclusion of the Barcelona-2000 EASL Conference European Association for the Study of the Liver. J Hepatol 2001;35:421.
5. Ryder S D. Guidelines for the diagnosis and treatment of hepatocellular carcinoma (HCC) in adults. Gut 2003;52:iii1.
6. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362:1907.
7. Balsells J, Charco R, Lazaro JL, et al. Resection of hepatocellular carcinoma in patients with cirrhosis. Br J Surg 1996;83:758.
8. Akamatsu K, Miyauchi S, Itoh Y, et al. Development and evaluation of a needle for percutaneous ethanol injection therapy. Radiology 1993;186:284.
9. Shiina S, Tagawa K, Unuma T, et al. Percutaneous ethanol injection therapy for hepatocellular carcinoma. A histopathologic study. Cancer 1991;68: 1524.
10. Livraghi T, Giorgio A, Marin G, et al. Hepatocellular carcinoma and cirrhosis in 746 patients: long-term results of percutaneous ethanol injection. Radiology 1995;197:101.
11. Seki T, Wakabayashi M, Nakagawa T, et al. Ultrasonically guided percutaneous microwave coagulation therapy for small hepatocellular carcinoma. Cancer 1994;74:817.
12. Murakami R, Yoshimatsu S, Yamashita Y, et al. Treatment of hepatocellular carcinoma: value of percutaneous microwave coagulation. Am J Roentgenol 1995;164:1159.
13. Rossi S, Buscarini E, Garbagnati F, et al. Percutaneous treatment of small hepatic tumours by an expandable RF needle electrode. Am J Roentgenol 1998;170:1015.
14. Goldberg SN, Gazelle GS, Solbiati L, et al. Ablation of liver tumours using percutaneous RF therapy. Am J Roentgenol 1998;170:1023.
15. Tateishi R, Shiina S, Teratani T, et al. Percutaneous radiofrequency ablation for hepatocellular carcinoma. An analysis of 1000 cases. Cancer 2005; 103:1201.
16. Horiike N, Iuchi H, Ninomiya T. Influencing factors for recurrence of hepatocellular carcinoma treated with radiofrequency ablation. Oncol Rep 2002;9:1059.
17. Solbiati L, Ierace T, Goldberg SN, et al. Percutaneous US-guided radio-frequency tissue ablation of liver metastases: treatment and follow-up in 16 patients. Radiology 1997;202:195.
18. Livraghi T, Goldberg SN, Monti F, et al. Saline-enhanced radio-frequency tissue ablation in the treatment of liver metastases. Radiology 1997;202: 582.
19. Berrington de Gonza'lez A, Darby S. Risk of from diagnostic X-rays; estimates for the UK and 14 other countries. Lancet 2004;363:345.
20. Shimizu M, Iijima H, Horibe T, et al. Usefulness of contrast enhanced ultrasonography with a new contrast mode, agent detection imaging, in evaluating therapeutic response in hepatocellular carcinoma treated with radio-frequency ablation therapy. Hepatol Res 2004;29:235.
21. Hirooka M, Iuchi H, Kurose K, et al. Abdominal virtual ultrasonographic images reconstructed by multi-detector row helical computed tomography. Eur J Radiol 2005;53:312.
22. Hirooka M, Iuchi H, Kumagi T, et al. Radiofrequency ablation using virtual ultrasonography of hepatocellular carcinoma visualized on CT not on conventional ultrasonography. Am J Roentgenol 2006;186:S255.
23. Kisaka Y, Hirooka M, Kumagi T, et al. Usefulness of contrast enhanced ultrasonography with abdominal virtual ultrasonography in assessing therapeutic response in hepatocellular carcinoma treated with radiofrequency ablation. Liver Int 2006;26:1241.
24. Koda M, Ueki M, Maeda Y, et al. Percutaneous sonographically guided radiofrequency ablation with artificial pleural effusion for hepatocellular carcinoma located under the diaphragm. Am J Roentgenol 2004;183:583.
25. Kim YS, Rhim H, Cho OK, et al. Intrahepatic recurrence after percutaneous radiofrequency ablation of hepatocellular carcinoma; analysis of the pattern and risk factor. Eur J Radiol 2006;59: 432.

26. Choi BI, Kim HC, Han JK, et al. Therapeutic effect of transcatheter oily chemoembolization therapy for encapsulated nodular hepatocellular carcinoma. CT and pathologic findings. Radiology 1992; 182:709.
27. Gazelle GS, Goldberg SN, Solbiati L, et al. Tumour ablation with radio-frequency energy. Radiology 2000;217:633.
28. Yamamoto K, Shiraki K, Nakanishi S, et al. 1.5 Harmonic imaging sonography with microbubble contrast agent improve characterization of hepatocellular carcinoma. World J Gastroenterol 2005;11: 5607.
29. Dutcher JP, Haney PJ, Whitley NO, et al. Ethiodized oil emulsion 13 in computed tomography of hepatoma. J Clin Oncol 1984;2:118.

Mass Reduction by Radiofrequency Ablation Before Hepatic Arterial Infusion Chemotherapy Improved Prognosis for Patients With Huge Hepatocellular Carcinoma and Portal Vein Thrombus

Masashi Hirooka[1]
Yohei Koizumi
Yoshiyasu Kisaka
Masanori Abe
Hidehiro Murakami
Bunzo Matsuura
Yoichi Hiasa
Morikazu Onji

OBJECTIVE. The prognosis for patients with advanced large hepatocellular carcinoma (HCC) with portal vein (PV) tumor thrombosis remains poor, and treatment is usually limited to hepatic arterial infusion (HAI) chemotherapy. In this study, we first performed mass reduction using radiofrequency ablation (RFA), followed by HAI chemotherapy. Prognosis after this treatment was evaluated.

SUBJECTS AND METHODS. HCC with PV tumor thrombosis was diagnosed in 20 patients between April 2004 and December 2008, and treatment was performed using mass-reduction therapy by RFA before HAI chemotherapy. For comparison, 33 patients treated with HAI chemotherapy without RFA were retrospectively selected as historical control subjects under the same conditions. Prognosis in each group was evaluated.

RESULTS. Mass-reduction therapy by RFA combined with HAI chemotherapy achieved complete response in six patients (30%), partial response in 11 patients (55%), stable disease in two patients (10%), and progressive disease in one patient (5%). Among the control subjects, complete response was seen in 0 patients (0%), partial response in 12 patients (33.3%), stable disease in 16 patients (44.4%), and progressive disease in eight patients (22.2%). The cumulative survival rates for those who received the combined therapy at 6, 12, and 24 months were 100%, 89.7%, and 78.8%, respectively. The median survival was 953 days (95% CI, 760–1,102 days). In the control subjects, the cumulative survival rates at 6, 12, and 24 months were 84.9%, 56.1%, and 16.9%, respectively ($p < 0.0001$). No serious adverse events were encountered in either group.

CONCLUSION. For patients with huge HCC and PV tumor thrombosis, mass-reduction treatment by RFA before HAI chemotherapy is safe and can improve prognosis.

Keywords: hepatic arterial infusion chemotherapy, hepatocellular carcinoma, portal vein tumor thrombosis, radiofrequency ablation

DOI:10.2214/AJR.09.2852

Received April 4, 2009; accepted after revision July 22, 2009.

[1]All authors: Department of Gastroenterology and Metabology, Ehime University Graduate School of Medicine, Shizukawa 454, Toon-shi, Ehime 791-0295, Japan. Address correspondence to Yoichi Hiasa (hiasa@m.ehime-u.ac.jp).

WEB
This is a Web exclusive article.

AJR 2010; 194:W221–W226

0361–803X/10/1942–W221

The prognosis is very poor for patients with hepatocellular carcinoma (HCC) invading the major branches of the portal vein (PV). PV tumor thrombus is associated with the threat of bleeding of the esophageal varices or hepatic failure. Thus, the patient's quality of life is poor.

In several studies, investigators have reported treatment of advanced HCC with PV tumor thrombosis using conventional treatments such as hepatectomy [1, 2], transcatheter hepatic arterial embolization [3], and chemotherapy [4–7]. With regard to aggressive treatment of HCC with PV tumor thrombosis, no acceptable outcomes have yet been obtained. Hepatic arterial infusion (HAI) chemotherapy has recently been reported to improve response rate and survival [4–6]. Although HAI chemotherapy has been attempted widely, the effectiveness of HAI chemotherapy remains unsatisfactory. Studies have revealed that adjuvant HAI chemotherapy after hepatectomy for HCC with PV tumor thrombosis is very effective [1, 8]. However, hepatectomy for small HCC is considered excessively invasive. Hepatectomy for advanced HCC certainly places a considerable burden on the patient. Conversely, radiofrequency ablation (RFA) is less invasive than hepatectomy. With developments in technique for RFA, large HCC and PV tumor thrombosis also can be treated by RFA [9–12].

We therefore propose a new method for the treatment of advanced HCC—using RFA for mass reduction of the tumor before HAI chemotherapy. The aim of this study was to evaluate the safety of this method and the prognosis of patients treated with this method compared with patients who had undergone conventional HAI chemotherapy without RFA.

Subjects and Methods

Patients and Inclusion Criteria

Twenty patients (16 men, four women; mean age, 63.9 ± 7.7 [SD] years) who had been admitted to the Department of Gastroenterology and Metabology of the Ehime University Hospital in Japan between April 2004 and December 2008 were diagnosed with HCC with PV tumor thrombosis. After we had obtained written, informed consent from study participants, mass-reduction therapy by RFA was performed before HAI chemotherapy. This prospective cohort study was conducted in accordance with the Declaration of Helsinki.

The criteria for study inclusion were as follows: first, Eastern Cooperative Oncology Group (ECOG) performance status of 0–2; second, successful implantation of an intraarterial catheter and drug delivery system; third, the existence of a giant nodule < 15 cm in diameter or of spread of tumor to comprise < 50% of the total liver volume; fourth, platelet count of > 70,000/mm^3; fifth, granulocyte count of > 2,500/mm^3; sixth, creatinine clearance of > 60 mL/h; and, seventh, the absence of extrahepatic metastasis. HCC was diagnosed on the basis of histologic findings; imaging studies; and elevated serum α-fetoprotein level, des-γ-carboxy prothrombin (DCP) level, or both.

Thirty-three patients (30 men, three women; mean age, 62.4 ± 7.8 [SD] years) had been treated using HAI chemotherapy without mass-reduction therapy by RFA between January 2002 and December 2008, and these patients were used as historical control subjects. Informed consent was obtained from all prospective subjects before they participated in the trial but was not obtained for retrospective data from historical control subjects. This study was approved by the ethics committee at Ehime University Hospital.

Catheter Implantation

Celiac angiography was performed using the femoral approach with the patient under local anesthesia. A 5-French heparin-coated catheter was introduced into the proper or common hepatic artery. The gastroduodenal artery and right gastric artery were occluded with steel coils to prevent gastroduodenal injury from anticancer agents. Aberrant hepatic arteries, if present, were occluded with metallic coils or a 1:1.5 mixture of *N*-butyl cyanoacrylate and iodized oil before treatment of hepatic arterial redistribution [13]. After the catheter was connected to the injection port, the device was implanted in a subcutaneous pocket in a femoral site. Patients received regional chemotherapy via the hepatic artery through a subcutaneously implanted port.

Treatment Protocol for Chemotherapy

We started the chemotherapy after the patients' complications (i.e., fever, elevation of transaminase level) from RFA had improved. Each patient received subcutaneous pegylated interferon (Peg-Intron, Schering-Plough Pharmaceuticals) and intraarterial infusion of 5-fluorouracil (5-FU Injection 250, Kyowa Hakko). One cycle of treatment lasted 4 weeks. Pegylated interferon (50 μg) was administered subcutaneously on days 1, 8, 15, and 22. Continuous infusion chemotherapy (300 mg/m^2/d of 5-fluorouracil) through the proper hepatic artery was administered in weeks 1 and 2 using the implanted drug-delivery system. A 2- or 3-week rest period separated each treatment cycle. All anticancer therapies were discontinued when adverse events reached level 2 according to ECOG classifications. At least two cycles of chemotherapy were performed.

Mass Reduction by RFA

Before mass-reduction treatment was performed, 15 mg of pentazocine hydrochloride and 25 mg of hydroxyzine hydrochloride were administered intramuscularly. Local anesthesia was induced using 5 mL of 1% lidocaine hydrochloride injected through the skin into the peritoneum along a predetermined puncture line. The radiologist performing the procedure inserted a 20-cm-long 17-gauge radiofrequency electrode equipped with a 2- or 3-cm-long exposed metallic tip (Cool-tip, Valleylab) or expandable needle (LeVeen Needle, Boston Scientific). If the diameter of the tumor was more than 5 cm, a 4-cm expandable needle was selected. The Cool-tip needle was used for the PV tumor thrombosis, in which the tumor had a diameter of less than 5 cm. This single needle also was used for residual lesion after ablating by LeVeen needle according to the protocol noted above. If the lung was obstructing the view of the nodule, 500 mL of saline was injected into the right pleural cavity [14].

Ablation was first performed for the intraparenchymal nodule, then the PV tumor thrombosis was treated. During the treatment of PV tumor thrombosis, RFA was performed first for the main portal branch, followed by ablation for PV tumor thrombosis in the peripheral branches. The electrode needle was inserted into the portal thrombus as close to the major axis of the PV tumor thrombosis as possible. A single needle was used to prevent injury to the major branches of the bile duct or artery. The electrode output was set as low as possible (i.e., < 50 W). If the hyperechoic area was not covered for the PV tumor thrombosis, an ethanol injection was added. Ethanol was injected until hyperechoic change of the target area could be seen. Usually, we injected 4 mL or less of ethanol for each session.

After the treatment, we evaluated the efficacy of RFA using CT. Complete necrosis was achieved for all ablated nodules except nonablated daughter nodules. We decided to perform the additional treatment if the viable portion remained by the time of the CT evaluation.

Estimation of Therapeutic Effect

Posttreatment evaluation was performed every 3 months. The evaluation mainly comprised periodic sonography; CT; and tests for tumor markers, including α-fetoprotein and DCP, for all patients. Objective response was classified according to ECOG criteria [15].

Statistical Analysis

Data are expressed as means ± SD. Statistical analysis was performed using the Student's *t* test for unpaired data, contingency table analysis, and the Mann-Whitney *U* test as appropriate. Cumulative survival curves were constructed using the Kaplan-Meier method. Values of $p < 0.05$ were considered to represent statistical significance.

Results

The Estimation of RFA

Only the Cool-tip needle was used for 20 cases, and both the Cool-tip needle and LeVeen needle were used for 14 cases. In no case was only the LeVeen needle used. Except tumor diameter, there was no significant difference between the cases treated using only the Cool-tip needle (47.4 ± 14.2 mm) and the cases treated using two needles (65.9 ± 22.1 mm) (p = 0.117). Ethanol injection was performed for four patients. The median volume of ethanol was 3.8 ± 0.3 mL (range, 3.5–4.2 mL). The mean number of RFA electrode punctures in each session was 3.0 ± 1.1 (range, 2–7). We ablated the small daughter nodules if there were two or more.

Post-RFA Complications

The mean number of ablation sessions was 1.7 ± 0.7 (range, 1–3). Complete necrosis of the PV thrombosis was achieved in 17 patients (85%). No serious adverse events were observed after ablation. No patients displayed liver abscess or bleeding, but all patients showed a transient elevation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The maximum values of AST and ALT after mass reduction by RFA were 221 and 251 IU/L, respectively. Elevation of ALT was mild (< 150 IU/L) in 10 patients and moderate (150–600 IU / L) in 10 patients. None of the patients showed severe ALT elevation (> 600 IU/L). The ALT level for all patients had recovered within 1 month after RFA. The median interval between the final

ablation until the start of chemotherapy was 9.1 ± 2.8 days (range, 5–14 days).

Clinical Response to Combination Therapy

Table 1 summarizes the characteristics of all the patients and control subjects. All patients completed at least two cycles of chemotherapy after RFA. For patients who displayed a clinical response, we continued chemotherapy until HCC progressed. If complete response was achieved, chemotherapy was stopped after the second cycle. Treatment was stopped after completion of the second cycle in patients with no response because of extended progression of HCC. The mean number of treatment cycles was 3.4 ± 2.2 (range, 2–10).

Among the 20 patients treated with RFA before HAI chemotherapy, six patients (30%) showed complete response; 11 (55%), partial response; two (10%), stable disease; and one (5%), progressive disease. In comparison, the control group showed complete response in 0 patients (0%), partial response in 12 (33.3%), stable disease in 16 (44.4%), and progressive disease in eight (22.2%) (Table 2). Of the 20 patients, six patients had PV tumor thrombosis in the main portal trunk and 14 in the major portal branch (Table 1). The response rate in these six patients who had PV tumor thrombosis in the major portal trunk was not significantly worse (83.3%) than that in the other 14 patients (response rate, 92.8%) ($p = 0.199$).

The response rate was significantly better for the mass-reduction group than for the control subjects ($p < 0.0001$). In four patients (20%), patency of the PV was achieved after combination treatment; in eight patients, partial PV patency was seen. Although the viable lesion in the PV tumor thrombosis had disappeared after treatment in two patients, PV flow did not resume in either patient, and cavernous transformation occurred. In the control group, complete patency of the PV occurred for 0 patients and partial patency, for seven patients. The repatency rate of the PV was significantly better ($p = 0.0016$) in the mass-reduction group than in the control group.

In the mass-reduction group, intrahepatic recurrence after chemotherapy was seen in three patients and extrahepatic recurrence, in five patients. In the control group, intrahepatic and extrahepatic recurrence was noted in eight and three patients, respectively ($p = 0.1016$). In the mass-reduction group, the cumulative survival rates at 6, 12, and 24 months were 100%, 89.7%, and 78.8%, respectively. The median survival time was 953 days (95% CI, 760–1,102 days). In the control group, the cumulative survival rates at 6, 12, and 24 months were 84.9%, 56.1%, and 16.9%, respectively. The median survival time was 352 days (95% CI, 267–407 days).

TABLE 1: Patient and Tumor Characteristics

Characteristic	Patients Who Received HAI Chemotherapy After RFA ($n = 20$)	Historical Control Subjects ($n = 33$)	p
Sex (no. of patients)			0.26
Male	16	30	
Female	4	3	
Age (y)			0.49
Mean ± SD	63.9 ± 7.7	62.4 ± 7.8	
Cause			0.76
Hepatitis B virus	5	6	
Hepatitis C virus	14	26	
Other[a]	1	1	
Child-Pugh class			0.72
A	16	25	
B	4	8	
Tumor diameter (mm)			0.40
Mean ± SD	57.3 ± 20.9	59.1 ± 28.5	
Extent of PV tumor thrombosis			0.26
Main portal trunk	6	15	
Major portal branch	14	18	

Note—HAI = hepatic arterial infusion, RFA = radiofrequency ablation, PV = portal vein.
[a]Non–hepatitis B virus, non–hepatitis C virus.

Overall survival rates were significantly improved for the mass-reduction group compared with control subjects ($p < 0.0001$, Fig. 1). The causes of death were as follows in the mass-reduction group: extension of cancer, nine patients; hepatic failure, none; gastroesophageal bleeding, two patients; and other cause, one patient. In the control group, the causes of death were extension of cancer, 27 patients; hepatic failure, two; gastroesophageal bleeding, one patient; and other cause, one patient ($p = 0.573$). Patients and control subjects died and tumor recurrences were noted during both periods of HAI chemotherapy and after cessation of HAI chemotherapy. The frequency between them was not different statistically.

Case Presentation

A 55-year-old man underwent RFA for HCC in January 2006. The largest HCC nodule (6.5 × 6.0 cm) in the right lobe was ablated using a 4.5-cm expandable needle. After mass reduction of the intrahepatic lesion, PV tumor thrombosis in the main and right portal branches was ablated using a single RFA needle. HAI chemotherapy (two cycles) was performed 11 days after the reduction therapy,

TABLE 2: Response to Hepatic Arterial Infusion (HAI) Chemotherapy in Patients Who Underwent Radiofrequency Ablation (RFA) and in Control Subjects Who Did Not

Response	Patients Who Received HAI Chemotherapy After RFA ($n = 20$)	Historical Control Subjects ($n = 36$)
Complete response	6	0
Partial response	11	12
Stable disease	2	16
Progressive disease	1	8
Response rate (%)	85.0	33.3
p	< 0.0001	

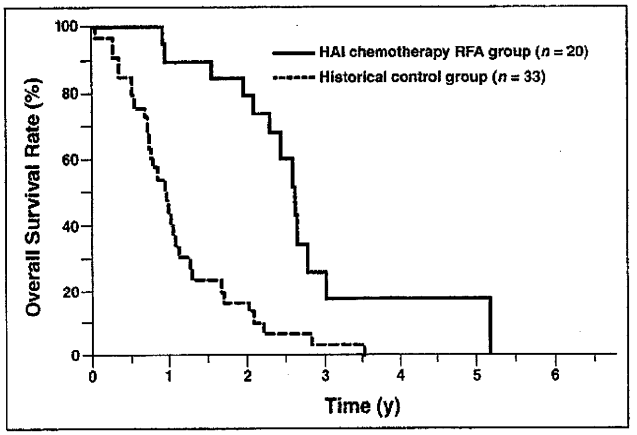


Fig. 1—Graph shows cumulative overall survival rates after hepatic arterial infusion (HAI) chemotherapy with and without radiofrequency ablation (RFA). Survival rate was higher for patients who underwent HAI chemotherapy and RFA than for control subjects who underwent HAI chemotherapy only ($p < 0.0001$).

and subsequent CT showed complete necrosis of tumor thrombosis. All tumor marker levels, which had been abnormal before treatment, normalized after RFA and HAI chemotherapy: α-fetoprotein level, from 580.4 to 2.5 ng/mL; α-fetoprotein-L3, from 43.1% to 0%; and DCP, from 598 to 27 mAU/mL (Fig. 2). This patient has undergone follow-up on an outpatient basis, with no evidence of recurrence as of the time of writing.

Discussion

The prognosis for patients with advanced HCC with PV tumor thrombosis remains poor [16]. The median survival time of HCC patients with PV tumor thrombosis is reportedly approximately 90 days with supportive care [17]. Many therapeutic modalities have been proposed to improve survival rate [1–8], but no standard treatment protocol has yet been defined.

HAI chemotherapy using implanted reservoirs has achieved significant survival benefits for patients with advanced HCC. Ishida et al. [4] reported that HAI chemotherapy with degradable starch microspheres (Spherex, Yakult) achieved an 84.6% response rate, with 1-, 2-, and 3-year survival rates of 100%, 28.9%, and 9.6%, respectively, in all patients and 100%, 33.3%, and 0% in patients with PV tumor thrombosis. The median survival was 22.1 months in all patients and 17.1 months in the six patients with PV tumor thrombosis [4].

If patients have a huge HCC mass with PV tumor thrombosis, response to treatment and survival are worsened [5]. Antitumor effects of those treatments against a huge mass of HCC are considered to be reduced for the following reasons: First, as HCC grows from the moderately differentiated type to the poorly or undifferentiated type, hypovascular changes occur, reducing the ability of anticancer drugs to be delivered to the advanced HCC. Second, huge HCC tumors are often located on the hepatic surface and are often fed by extrahepatic arteries, such as the adrenal or intercostal arteries [18, 19]. Again, this serves to reduce delivery of the anticancer drug from the hepatic artery. Third, in patients with huge HCC, particularly those with PV tumor thrombosis, portal flow is stagnated by invasion of the HCC, reducing liver function [16]. Moreover, delivery of anticancer drugs is again reduced in such cases. Given these factors, assistance by mass-reduction treatment seemed likely to prove useful for patients with huge HCC with PV tumor thrombosis and could improve prognosis compared with HAI chemotherapy monotherapy.

Mass-reduction therapy for patients with huge HCC nodules and PV tumor thrombosis could be compared with transcatheter arterial embolization (TAE) or surgical resection. TAE is a useful method for treating huge nodules. However, TAE requires injection of embolization material through a catheter into the hepatic artery. The response of huge HCC to TAE is often insufficient because of the reasons mentioned earlier. Moreover, if cavernous transformation of the PV has not occurred in cases of HCC with PV tumor thrombosis, parenchymal infarction around HCC can result from TAE, further damaging the liver. TAE is unsuitable for mass-reduction treatment of huge HCC.

Recently, the results of ^{90}Y therapy for huge HCC have been reported [20]. In Japan, we cannot use ^{90}Y therapy. Because ^{90}Y therapy is radiochemoembolization, ^{90}Y therapy is not suitable for treatment of recurrences. Riaz et al. [20] reported that the rate of complete necrosis by the treatment of ^{90}Y was only 17% of the tumor for tumors more than 5 cm in diameter, whereas complete necrosis was achieved in all nodules more than 5 cm using our RFA method.

The most curative approach to treating huge HCC with PV tumor thrombosis is surgical resection. However, this option is feasible in only a minority of HCC patients because most patients with huge HCC with PV tumor thrombosis display seriously compromised liver function. Abdominal open surgery is thus too invasive for patients with a limited prognosis. Local ablation therapy for huge HCC has recently been reported [9, 10]. Wider lesions of the liver can be ablated using a multipolar needle and large expandable needle.

For the current study, we performed mass-reduction treatment using RFA and tried to treat PV tumor thrombosis aggressively. In our study, four patients with Child-Pugh grade B cirrhosis showed no deterioration of liver function after ablation of wide HCC lesions. Livraghi et al. [21] reported their first experience of treating PV tumor thrombosis using percutaneous ethanol injection (PEI). Conversely, Giorgio et al. [11] reported treatment of PV tumor thrombosis using RFA.

For the treatment of PV tumor thrombosis, RFA seems more risky than PEI. The risk of bleeding is thought to be higher because the RFA needle that is inserted into the PV is thicker than the PEI needle. Moreover, PVs accompany bile ducts and the hepatic arteries in the liver. If bile ducts and hepatic arteries are injured by RFA for PV tumor thrombosis, severe complications such as biloma, obstructive jaundice, liver abscess, and mass liver infarction can result. We performed RFA for PV tumor thrombosis with a low output (< 50 W), and none of these complications occurred in any of the patients in our study group.

Giorgio et al. [11] reported 10 cases of advanced HCC with PV tumor thrombosis treated using RFA (mean tumor diameter, 4.20 ±

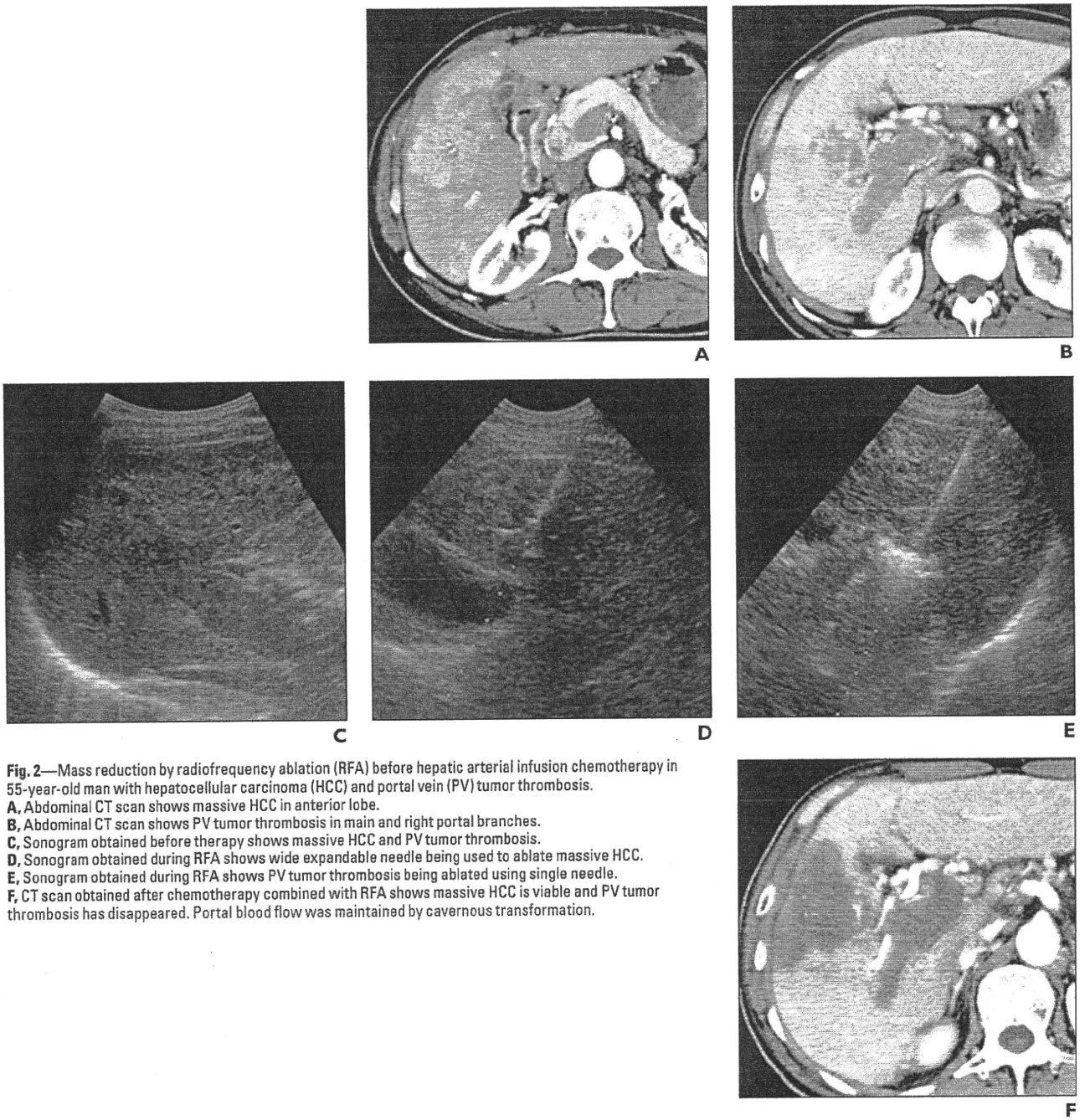


Fig. 2—Mass reduction by radiofrequency ablation (RFA) before hepatic arterial infusion chemotherapy in 55-year-old man with hepatocellular carcinoma (HCC) and portal vein (PV) tumor thrombosis.
A, Abdominal CT scan shows massive HCC in anterior lobe.
B, Abdominal CT scan shows PV tumor thrombosis in main and right portal branches.
C, Sonogram obtained before therapy shows massive HCC and PV tumor thrombosis.
D, Sonogram obtained during RFA shows wide expandable needle being used to ablate massive HCC.
E, Sonogram obtained during RFA shows PV tumor thrombosis being ablated using single needle.
F, CT scan obtained after chemotherapy combined with RFA shows massive HCC is viable and PV tumor thrombosis has disappeared. Portal blood flow was maintained by cavernous transformation.

0.36 cm; range, 3.8–4.9 cm). Although the grade of HCC was more serious in our study, our results in terms of survival time (6-, 12-, and 24-month rates of 100%, 89.7%, and 78.8%, respectively) were also good. In four cases in which ablation by RFA was insufficient, we performed PEI for the treatment of PV tumor thrombosis. In all four patients, complete PV flow resumed (Fig. 3).

In some patients, the RFA needle may not reach the far side of the liver where PV tumor thrombosis has expanded to the superior mesenteric vein or splenic vein. Fortunately, we did not encounter any such cases in this study. However, radiotherapy should be considered in such cases.

The effects of radiation associated with mass-reduction treatment and HAI chemotherapy need to be evaluated in a future study. More or less arterial–portal shunting was noted by angiography in all patients who had the PV tumor thrombosis. Our results of the treatment in the patients who had arterial–portal shunting was not bad, indicating that the arterial–portal shunting might not induce the attenuation of the effect of chemotherapy.

In conclusion, we propose a less invasive combined technique using mass-reduction

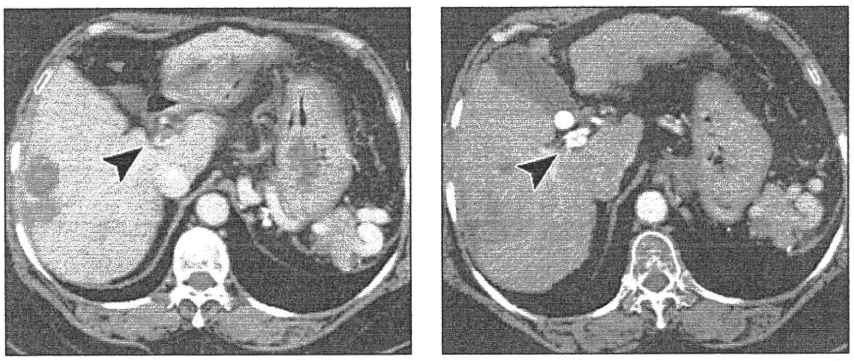


Fig. 3—Improvement of portal blood flow in 72-year-old woman with portal vein (PV) tumor thrombosis who underwent hepatic arterial infusion chemotherapy after radiofrequency ablation.
A, CT scan obtained before patient underwent combined therapy shows lumen of portal major trunk is occluded by PV tumor thrombosis (*arrowhead*).
B, CT scan obtained after patient received combined therapy shows blood flow of portal major trunk (*arrowhead*) has recovered.

treatment by RFA and HAI chemotherapy for the treatment of huge HCC and PV tumor thrombosis. This treatment appears safe and beneficial for patients showing huge HCC with PV tumor thrombosis, improving prognosis.

References

1. Fukuda S, Okuda K, Imamura M, Imamura I, Eriguchi N, Aoyagi S. Surgical resection combined with chemotherapy for advanced hepatocellular carcinoma with tumor thrombus: report of 19 cases. *Surgery* 2002; 131:300–310
2. Inoue Y, Hasegawa K, Ishizawa T, et al. Is there any difference in survival according to the portal tumor thrombectomy method in patients with hepatocellular carcinoma? *Surgery* 2009; 145:9–19
3. Chung JW, Park JH, Han JK, Choi BI, Han MC. Hepatocellular carcinoma and portal vein invasion: result of treatment with transcatheter oily chemoembolization. *AJR* 1995; 165:315–321
4. Ishida K, Hirooka M, Hiraoka A, et al. Treatment of hepatocellular carcinoma using arterial chemoembolization with degradable starch microspheres and continuous arterial infusion of 5-fluorouracil. *Jpn J Clin Oncol* 2008; 38:596–603
5. Sakon M, Nagano H, Dono K, et al. Combined intraarterial 5-fluorouracil and subcutaneous interferon-alpha therapy for advanced hepatocellular carcinoma with tumor thrombi in the major portal branches. *Cancer* 2002; 94:435–442
6. Obi S, Yoshida H, Toune R, et al. Combination therapy of intra-arterial 5-fluorouracil and systemic interferon alpha for advanced hepatocellular carcinoma with portal venous invasion. *Cancer* 2006; 106:1990–1997
7. Ando E, Yamashita F, Tanaka M, et al. A novel chemotherapy for advanced hepatocellular carcinoma with tumor thrombosis of the main trunk of the portal vein. *Cancer* 1997; 79:1890–1896
8. Nagano H, Miyamoto A, Wada H, et al. Interferon alpha and 5-fluorouracil combination therapy after palliative hepatic resection in patients with advanced hepatocellular carcinoma, portal venous tumor thrombus in the major trunk, and multiple nodules. *Cancer* 2007; 110:2493–2501
9. Takaki H, Yamakado K, Uraki J, et al. Radiofrequency ablation combined with chemoembolization for the treatment of hepatocellular carcinomas larger than 5 cm. *J Vasc Interv Radiol* 2009; 20:217–224
10. Chen MH, Yang W, Zou MW, Solbiati L, Liu JB, Dai Y. Large liver tumors: protocol for radiofrequency ablation and its clinical application in 110 patients—mathematic model, overlapping mode, and electrode placement process. *Radiology* 2004; 232:260–271
11. Giorgio A, Stefano G, Sarno A, et al. Radiofrequency ablation of hepatocellular carcinoma extended into the portal vein: preliminary results. *J Ultrasound* (in press)
12. Zheng RQ, Kudo M, Minami Y, Inui K, Chung H. Stage IV hepatocellular carcinoma with portal venous tumor thrombus: complete response after combined therapy of repeated arterial chemoembolization and radiofrequency ablation. *J Gastroenterol* 2003; 38:406–409
13. Stoesslein F, Ditscherlein G, Romaniuk PA. Experimental studies on new liquid embolization mixtures (Histoacryl-Lipiodol, Histoacryl-Pantopaque). *Cardiovasc Intervent Radiol* 1982; 5: 264–267
14. Uehara T, Hirooka M, Ishida K, et al. Percutaneous ultrasound-guided radiofrequency ablation of hepatocellular carcinoma used with artificially-induced pleural effusion and ascites. *J Gastroenterol* 2007; 42:306–311
15. Oken MM, Creech RH, Tormey DC, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol* 1982; 5: 649–655
16. Kudo M, Chung H, Haji S, et al. Validation of a new prognostic staging system for hepatocellular carcinoma: the JIS score compared with the CLIP score. *Hepatology* 2004; 40:1396–1405
17. Takizawa D, Kakizaki S, Sohara N, et al. Hepatocellular carcinoma with portal vein tumor thrombosis: clinical characteristics, prognosis, and patient survival analysis. *Dig Dis Sci* 2007; 52:3290–3295
18. Miyama S, Matsui O, Taki K, et al. Extrahepatic blood supply to hepatocellular carcinoma: angiographic demonstration and transcatheter arterial chemoembolization. *Cardiovasc Intervent Radiol* 2006; 29:39–48
19. Park SI, Lee DY, Won JY, Lee JT. Extrahepatic collateral supply of hepatocellular carcinoma by the intercostal artery. *J Vasc Interv Radiol* 2003; 14:461–468
20. Riaz A, Kulik L, Lewandowski RJ, et al. Radiologic–pathologic correlation of hepatocellular carcinoma treated with internal radiation using yttrium-90 microspheres. *Hepatology* 2009; 49: 1185–1193
21. Livraghi T, Grigioni W, Mazziotti A, Sangalli G, Vettori C. Percutaneous alcohol injection of portal thrombosis in hepatocellular carcinoma: a new possible treatment. *Tumori* 1990; 76:394–397

Antiviral Therapy 2010 15:887-895 (doi: 10.3851/IMP1637)

Original article

Immune modulator and antiviral potential of dendritic cells pulsed with both hepatitis B surface antigen and core antigen for treating chronic HBV infection

Sheikh Mohammad Fazle Akbar[1,2], Osamu Yoshida[2], Shiyi Chen[2], Aguilar Julio Cesar[3], Masanori Abe[2], Bunzo Matsuura[2], Yoichi Hiasa[2], Morikazu Onji[2]*

[1]Department of Medical Sciences, Toshiba General Hospital, Tokyo, Japan
[2]Department of Gastroenterology and Metabology, Ehime University Graduate School of Medicine, Ehime, Japan
[3]Vaccine Division, Center for Genetic Engineering and Biotechnology, Havana, Cuba

*Corresponding author e-mail: sheikh.akbar@po.toshiba.co.jp

Background: Commercially available prophylactic vaccines containing hepatitis B surface antigen (HBsAg), which are used to prevent HBV infections, are not as effective as a therapeutic immune modulator for treating patients with chronic hepatitis B (CHB). In this study, the immunogenicity of dendritic cells (DC) loaded with both HBsAg and hepatitis B core antigen (HBcAg) was tested in HBV transgenic mice (TM; 1.2HB-BS10) *in vivo* and in patients with CHB *in vitro*.

Methods: Spleen DC from HBV TM were cultured with a vaccine containing both HBsAg and HBcAg to produce HBsAg/HBcAg-pulsed DC. HBV TM were immunized twice at an interval of 4 weeks with HBsAg/HBcAg-pulsed DC and other immune modulators. Antibody titres to HBsAg (anti-HBs) were measured in sera. Antigen-specific T-cells and cytotoxic T-lymphocytes (CTLs) in the spleen and liver were detected by lymphoproliferative and ELISPOT assays, respectively. HBsAg/HBcAg-pulsed human blood DC were cultured with autologous T-cells from CHB patients to assess their antigen-specific immune modulatory capacities.

Results: Significantly higher levels of anti-HBs, HBsAg-specific and HBcAg-specific T-cells and CTLs were detected in the spleen and liver of HBV TM immunized with HBsAg/HBcAg-pulsed DC compared with those immunized with other vaccine formulations ($P<0.05$). HBsAg/HBcAg-pulsed human blood DC also induced HBsAg- and HBcAg-specific proliferation of autologous T-cells from CHB patients.

Conclusions: The immune modulatory capacities of HBsAg/HBcAg-pulsed DC in HBV TM *in vivo*, and in patients with CHB *in vitro*, inspire optimism about a clinical trial with this cell-based vaccine in patients with CHB.

Introduction

Important insights about epidemiology, virology, immunology and pathogenesis of HBV have been documented during the past four decades. However, no curative therapy against chronic hepatitis B (CHB) has been developed. Clinical trials with different antiviral drugs (type-I interferon [IFN] and nucleoside/nucleotide analogues) have inspired considerable optimism about their use in CHB patients on the basis of intermediate outcomes [1]. However, a systemic review of a National Institutes of Health Consensus Development Conference that analysed all randomized clinical trials (RCTs) with antiviral drugs in CHB patients from 1989 to 2008 showed that drug treatment did not improve ultimate clinical outcomes or all intermediate outcomes in any RCT [2]. Low and moderate quality RCTs suggested improvement of some intermediate parameters by using antiviral drugs in CHB patients [2]. However, these drugs are costly, might require prolonged use, and are associated with several side effects, including the emergence of treatment-induced mutant HBV. These findings indicate that alternative therapeutic approaches should be developed to treat CHB.

The concept of immune therapy as an alternative therapeutic approach for treating CHB patients has emerged for several reasons. First, patients with CHB exhibit distorted immune responses to various HBV-related antigens [3]. In addition, sustained control of HBV replication and liver damage in CHB patients is usually associated with restoration of host immunity [4]. However, the therapeutic efficacy of polyclonal immune

modulators, such as immune IFN, growth factors and cytokines, was not satisfactory in CHB patients [5]. Subsequently, therapeutic vaccines containing hepatitis B surface antigen (HBsAg) have been used since the early 1990s. However, it is unlikely that the present vaccines will be able to stand the test of time [6].

We found some limitations to the current therapeutic vaccines for CHB patients. First, HBsAg is administered to CHB patients with the assumption that it will be internalized, processed and presented by antigen-presenting cells to induce HBsAg-specific immune responses [7]. However, because the phenotypes and functions of dendritic cells (DC), the most potent antigen-presenting cells, are distorted in CHB [8], it is unlikely that the DC of CHB patients would be able to properly process and present HBsAg for restoration of HBV-specific immunity. Second, vaccine therapies in CHB patients have been accomplished with HBsAg-based vaccines only. However, both HBsAg and hepatitis B core antigen (HBcAg)-specific immune responses are essential for sustained control of HBV replication and containment of liver damage [3]. Third, almost all patients with CHB harbour considerable levels of HBsAg; thus, μg levels of HBsAg in commercial vaccines might not be sufficient to overcome the immune tolerance state of these patients. In addition, clonal deletion of HBV-specific T-cells and the exhaustion of antiviral cytotoxic T-lymphocytes (CTLs) by high doses of antigen might also have a role in the minimal effect of HBsAg-based vaccine therapy [9]. Taken together, these factors indicate that a better regimen of immune therapy against HBV might be created by delivering HBV-related antigens with adequately activated antigen-presenting DC in CHB patients.

The immune modulator effects of HBsAg-pulsed DC have been evaluated in HBV transgenic mice (TM) by other investigators, as well as our group [10,11]. Jiang *et al.* [10] have shown that immunization with peptide-pulsed DC could elicit antiviral immunity in HBV TM. However, they used a murine model of HBV that expressed only HBsAg; therefore, the clinical implications of their study for translation research in patients with CHB are limited [10]. We found that HBsAg-pulsed DC induced antibody to HBsAg (anti-HBs) production, but not HBsAg-specific cellular immunity, in HBV TM [11].

Availability of a human consumable vaccine containing both HBsAg and HBcAg led us to perform the present study. First, we loaded spleen DC from normal C57BL/6J mice with this vaccine to optimize culture conditions. Then, we assessed the specificity and immune modulatory functions of HBsAg/HBcAg-pulsed DC *in vitro*. Subsequently, a preclinical study was conducted in HBV TM with HBsAg/HBcAg-pulsed DC and several other immune modulators. Finally, we extended this study to evaluate whether HBsAg/HBcAg-pulsed human blood DC from CHB patients were capable of activating autologous immunocytes in an antigen-specific manner *in vitro*. The research potential of this study will be discussed for developing antigen-specific immune therapy against chronic HBV infection in humans.

Methods

Mice

HBV TM (official designation 1.2HB-BS10) were prepared by microinjecting the complete genome of HBV plus 619 base pairs (bp) of HBV DNA into the fertilized eggs of C57BL/6 mice. The HBV TM expressed HBV DNA and messenger RNA of 3.5, 2.1 and 0.8 kbp of HBV in the liver. HBV DNA were also detected in the liver and sera of HBV TM. HBsAg was found in the sera of all HBV TM [12]. Male C57BL/6J mice that were 8 weeks old were purchased from Nihon Clea Co. (Tokyo, Japan). Mice were housed in polycarbonate cages in our laboratory facilities and maintained in a temperature- and humidity-controlled room (23 ±1°C) with a 12-h light/dark cycle. All mice received humane care, and the study protocol was approved by the Ethics Committee of the Graduate School of Medicine, Ehime University (Ehime, Japan).

Patients with CHB

Peripheral blood mononuclear cells and DC were isolated from five patients with clinical, biochemical and histological evidence of CHB. They were attending Ehime University Hospital (Ehime, Japan) for regular follow-up. The mean ±SD age of the patients was 35 ±12 years and the level of alanine aminotransferase was 127 ±32 IU/l (normal range 5–48 IU/l). Liver biopsy revealed a moderate degree of activity of hepatitis and moderate levels of fibrosis. Patients had not taken any antiviral or immune modulator drugs during the previous 6 months. Informed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the Ehime University Hospital human research committee.

Detection of HBV-related markers

Levels of HBsAg and anti-HBs in sera were estimated using a chemiluminescence enzyme immunoassay (Special Reference Laboratory, Tokyo, Japan) and were expressed as IU/ml and mIU/ml, respectively [13]. Antibodies to HBcAg in sera were estimated by the passive hemagglutination method (Tokyo Institute of Immunology, Tokyo, Japan). HBV DNA in the sera of HBV TM was assessed by the PCR method (Special Reference Laboratory).

Isolation of T-cells, B-cells and DC

The methodologies for isolating spleen cells, T-cells, B-cells and DC are described in detail elsewhere [14]. In brief, spleens were removed aseptically, cut into pieces and incubated at 37°C in 5% CO_2 for 30 min in RPMI 1640 (Nipro, Osaka, Japan) supplemented with 1 µg/ml collagenase (type IV; Sigma Aldrich Corporation, St Louis, MO, USA), and a single-cell suspension of spleen was produced. T-cells were purified from single-cell suspensions of spleen by a negative-selection column method (Mouse Pan T Isolation Kit; Miltenyi Biotec, Bergisch Gladbach, Germany). $CD8^+$ T-cells were purified from T-cells by a column method (Mouse CD8 Isolation Kit; Miltenyi Biotec).

To isolate spleen DC, single-cell suspension of spleen was centrifuged at 10,000×*g* for 30 min on a dense albumin column (specific gravity 1.082) at 4°C and then cultured on a plastic surface for 90 min at 37°C. The adherent cells were cultured for an additional 18 h in culture medium containing RPMI 1640 plus 10% fetal calf serum (Filtron PTY Ltd, Brooklyn, Australia). Macrophages were discarded from DC populations by two additional adherent steps on plastic dishes at 37°C.

Liver non-parenchymal cells (NPC) were isolated as described previously [15]. In brief, liver tissues were homogenized, suspended in 35% percoll (Sigma Aldrich Corporation), and centrifuged to get liver NPC. Liver NPC were suspended in RPMI 1640 plus 10% fetal calf serum.

Human blood DC were enriched from peripheral blood of CHB patients by culturing them with granulocyte macrophage colony-stimulating factor and interleukin (IL)-4 for 6 days, as described previously [16].

Preliminary experiments to optimize culture conditions for preparing immunogenic antigen-pulsed DC

Normal C57BL/6J mice were immunized with HBsAg (10 µg; Tokyo Institute of Immunology), HBcAg (10 µg; Tokyo Institute of Immunology), HBsAg/HBcAg (10 µg Center for Genetic Engineering and Biotechnology [CIGB], Havana, Cuba) and pyruvate dehydrogenase complex (PDC; 10 µg; Sigma Aldrich Corporation) [17] in phosphate-buffered saline (PBS) twice at an interval of 4 weeks. Lymphocytes from immunized mice were cultured with antigen-pulsed DC from non-immunized mice to evaluate if antigen-pulsed DC could induce proliferation of antigen-specific lymphocytes *in vitro*.

Preparation of antigen-pulsed DC for immunization of HBV TM

HBsAg, HBcAg and HBsAg/HBcAg, used for the assessment of therapeutic efficacy of antigen-pulsed DC in HBV TM, were provided by the CIGB [18]. *Pichia-pastoris*-derived recombinant HBsAg was used. HBcAg were derived from *Escherichia coli* purified recombinant full-length HBcAg (GenBank accession number X02763). HBsAg/HBcAg consisted of equal amounts of HBsAg and HBcAg. HBsAg was produced as a 22 nm particle to >95% purity at the CIGB production facilities as a component of the commercial anti-HBV vaccine, Heberbiovac-HB1. HBcAg was purified from *E. coli* strain W3110, which had been transformed previously with a plasmid containing the entire core antigen gene under the control of a tryptophan promoter. The resulting HBcAg had a purity >95% [18].

Murine antigen-pulsed DC were prepared based on data from preliminary studies and also according to our previous report [19]. Briefly, spleen DC were cultured with PDC (10 µg), HBsAg (10 µg), HBcAg (10 µg) and HBsAg/HBcAg vaccine (HBsAg 5 µg and HBcAg 5 µg) in culture medium for 48 h. DC were recovered from the cultures and washed 5× with PBS. The viability of DC was assessed by the trypan blue exclusion test. The T-cell stimulatory capacity of antigen-pulsed DC were confirmed by lymphoproliferative assays. Human blood DC was cultured with HBsAg/HBcAg vaccine (HBsAg 5 µg and HBcAg 5 µg) or PDC (10 µg) for 48 h to prepare antigen-pulsed DC, as described elsewhere [16].

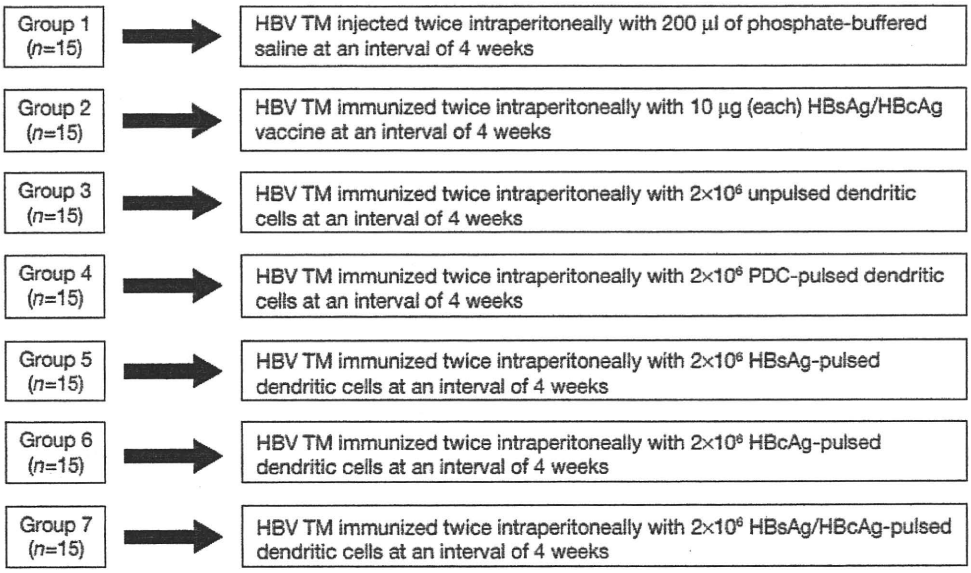
Immunization schedule

The immunization schedule is shown in Figure 1. Seven groups of HBV TM with comparable levels of HBsAg in the sera were used for this preclinical study. One group of HBV TM was injected with PBS (n=15). A second and third group of HBV TM received the HBsAg/HBcAg vaccine (10 µg; n=15) and 2×10^6 unpulsed DC (n=15), respectively. A fourth, fifth and sixth group of HBV TM received 2×10^6 PDC-pulsed DC (n=5), 2×10^6 HBsAg-pulsed DC (n=15) and 2×10^6 HBcAg-pulsed DC (n=15). Finally, a seventh group of HBV TM received HBsAg/HBcAg-pulsed DC (n=15). All vaccinations were done by the intraperitoneal route, twice at an interval of 4 weeks. HBV TM were bled from the tail vein at different intervals to assess different immunological parameters. The mice were finally euthanized to estimate vaccine-induced cellular immune responses in the spleen and liver.

Lymphoproliferative assays

As described previously, murine lymphocytes, murine liver NPC and human peripheral blood mononuclear cells were cultured in the absence or presence of different immune modulators for 120 h to evaluate antigen-specific cellular immune responses [14–17,19,20]. All cultures were performed in 96-well U-bottom plates (Corning Inc., New York, NY, USA). [^{3}H]-thymidine (1.0 µCi/ml; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) was diluted in sterile PBS, added to the cultures for the last 16 h and harvested automatically by a multiple cell harvester (Labo Mash;

Figure 1. Immunization schedule for assessment of antigen-specific humoral and cellular immune responses in HBV TM



HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; PDC, pyruvate dehydrogenase complex; TM, transgenic mice.

Futaba Medical, Osaka, Japan) onto a filter paper (LM 101–10; Futaba Medical). The levels of incorporation of [^{3}H]-thymidine were determined in a liquid scintillation counter (Beckman LS 6500; Beckman Instruments, Inc., Fullerton, CA, USA) from the level of blastogenesis. Triplicate cultures were assayed routinely and the results were expressed as cpm. The stimulation index was calculated as the ratio of cpm obtained in the presence of antigen or antigen-pulsed DC to that obtained without antigen or in presence of only DC or irrelevant antigen-pulsed DC. A stimulation index >3.0 was considered significant.

ELISPOT assays

Different types of immunocytes (1×10^5) were stimulated with antigen in presence of mitomycin-C-treated spleen adherent cells in an IFN-γ-coated ELISPOT plate (Mabtech, Nacka Strand, Sweden) for 24 h. After removing the cells, biotinylated antibody (2A5-biotin) was added into the wells. After 2 h incubation, the plates were further incubated with streptavidin–alkaline phosphatase for 1 h. After washing the plates, the substrate solution, BCIP/NBT, was added. The reaction was stopped by washing the plates extensively with tap water. The numbers of spot-forming units were counted using an ELISPOT reader (KS ELISPOT; Carl Zeiss, Thornwood, NY, USA). Different types of immunocytes were stimulated with concanavalin A as a positive control.

Statistical analyses

Data are shown as mean ±SD. Means were compared using the Student's *t*-test. For differences determined by the F-test, the Student's *t*-test was adjusted for unequal variances (Mann–Whitney U test). $P<0.05$ was considered statistically significant.

Results

Features of antigen-pulsed DC and their functional capacities

We first isolated DC from normal C57BL/6 mice spleen and human blood. Murine spleen DC expressed major histocompatibility complex (MHC) class II antigen and CD86 antigens. Human monocyte-derived DC expressed human leukocyte antigen DR and CD86 antigens. A functional study showed that both murine spleen DC and human blood DC induced proliferation of allogenic T-cells in a dose-dependent manner (SMFA *et al.*, data not shown).

Antigen-pulsed DC from normal C57BL/6 mice produced significantly higher levels of IL-12 (HBsAg-pulsed DC 154.3 ±12.3 pg/ml and HBcAg-pulsed DC 213.2 ±23.6 pg/ml; $n=3$) compared with unpulsed DC (35.3 ±9.6 pg/ml; $n=3$; $P<0.05$). Also, HBsAg-pulsed and HBcAg-pulsed DC induced proliferation of lymphocytes from HBsAg- and HBcAg-immunized normal C57BL/6J mice (SMFA *et al.*, data not shown). After

optimizing culture conditions for preparing immunogenic HBsAg-pulsed DC and HBcAg-pulsed DC, we prepared HBsAg/HBcAg-pulsed DC from normal C57BL/6J mice. HBsAg/HBcAg-pulsed DC expressed significantly higher levels of MHC class II and CD86 compared to unpulsed DC ($P<0.05$). Also, HBsAg/HBcAg-pulsed DC produced significantly higher levels of IL-12 and IFN-γ compared with unpulsed DC ($P<0.05$). As shown in Table 1, HBsAg/HBcAg-pulsed DC did not induce significant proliferation of lymphocytes from PDC-immunized normal C57BL/6J mice (stimulation index 1.0), but induced vigorous proliferation of lymphocytes from HBsAg/HBcAg-immunized normal C57BL/6J mice (stimulation index 17.3 ±3.2; $n=3$). By contrast, PDC-pulsed DC induced significant proliferation of lymphocytes from PDC-immunized normal C57BL/6J mice (stimulation index 9.6 ±2.2; $n=3$). However, PDC-pulsed DC did not induce proliferation of HBsAg/HBcAg-immunized normal C57BL/6 mice (stimulation index 1.0). After assessment of immunogenecity of antigen-pulsed DC in normal C57BL/6 mice *in vitro*, we next evaluated immunogenecity of antigen-pulsed DC in HBV TM *in vivo*.

HBsAg and anti-HBs in HBV TM immunized with antigens and antigen-pulsed DC

When the levels of HBsAg were estimated 4 weeks after the second immunization, the levels of HBsAg in the sera were decreased or became undetectable in HBV TM immunized with HBsAg-pulsed DC and HBsAg/HBcAg-pulsed DC. However, there was no significant alteration in HBsAg levels in HBV TM immunized with other formulations (Figure 2A).

Anti-HBs were not detected in PBS-injected HBV TM ($n=15$). In addition, anti-HBs were not detected in HBV TM injected twice with vaccine containing HBsAg/HBcAg ($n=15$) or unpulsed DC ($n=15$), PDC-pulsed DC ($n=5$) or HBcAg-pulsed DC ($n=15$). However, anti-HBs were detected in all HBV TM after two injections with HBsAg/HBcAg-pulsed DC (Figure 2B). Anti-HBs were also detected in HBV TM immunized with HBsAg-pulsed DC, but the levels of anti-HBs in these HBV TM were significantly lower than those in HBV TM immunized with HBsAg/HBcAg-pulsed DC (Figure 2B).

Antigen-specific cellular immune responses caused by vaccination with HBsAg/HBcAg-pulsed DC

We checked antigen-specific cellular immune responses in different groups of HBV TM. Lymphocytes from HBV TM immunized with HBsAg/HBcAg-pulsed DC proliferated in response to both HBsAg and HBcAg (Figure 3). However, lymphocytes from PBS-injected HBV TM, HBsAg/HBcAg vaccine-immunized HBV TM, unpulsed DC-injected HBV TM and PDC-pulsed DC-immunized HBV TM did not proliferate because of stimulation with HBsAg or HBcAg. Lymphocytes from HBV TM immunized with HBsAg-pulsed DC and HBcAg-pulsed DC proliferated in response to respective antigens only, but not to both HBsAg and HBcAg (Figure 3).

These data revealed that immunization with HBsAg/HBcAg-pulsed DC was capable of inducing HBsAg-specific humoral, HBsAg-specific cellular and HBcAg-specific cellular immune responses in HBV TM. Next, we checked if antigen-specific CTLs were produced in the spleen as a result of immunization of HBV TM with HBsAg/HBcAg-pulsed DC.

Detection of IFN-γ producing CTL in HBV TM immunized with HBsAg/HBcAg-pulsed DC

Spleen T-cells from HBV TM injected with HBsAg/HBcAg-pulsed DC, but not those from HBV TM immunized with HBsAg/HBcAg vaccine, unpulsed DC or PBS, or PDC-pulsed DC, produced significant numbers of IFN-γ-secreting T-cells in response to stimulation with HBsAg and HBcAg in the ELISPOT assay (Table 2). Also, abundant numbers of IFN-γ-secreting T-cells were detected in ELISPOT assays when spleen T-cells were stimulated with concanavalin A (positive control; SMFA *et al.* data not shown).

Antigen-specific immunocytes in the liver resulting from immunization with HBsAg/HBcAg-pulsed DC

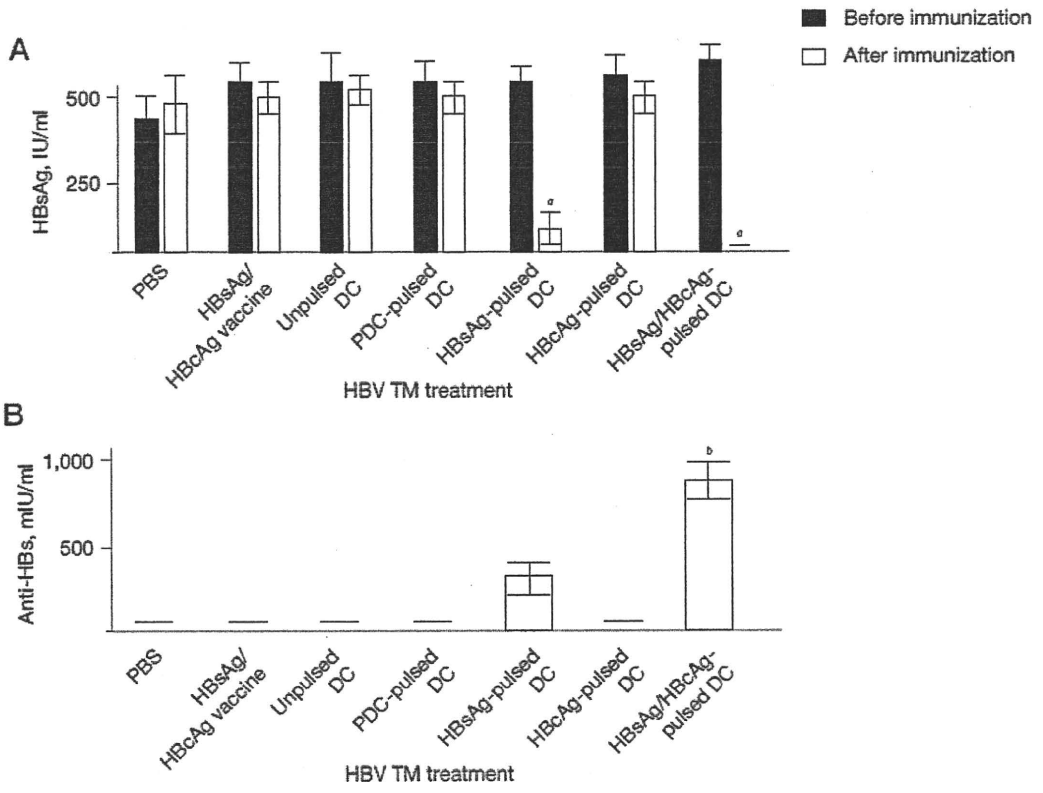
Although antigen-specific lymphocytes and CTLs were detected in the spleen, it was important to assess if antigen-specific lymphocytes and CTLs were induced

Table 1. Antigen-specific proliferative capacities of antigen-pulsed dendritic cells

Lymphocyte	DC	Stimulation index
HBsAg/HBcAg-immunized mice	PDC-pulsed	1.0
HBsAg/HBcAg-immunized mice	HBsAg/HBcAg-pulsed	17.3 ±3.2
PDC-immunized mice	HBsAg/HBcAg-pulsed	1.0
PDC-immunized mice	PDC-pulsed	9.6 ±2.2

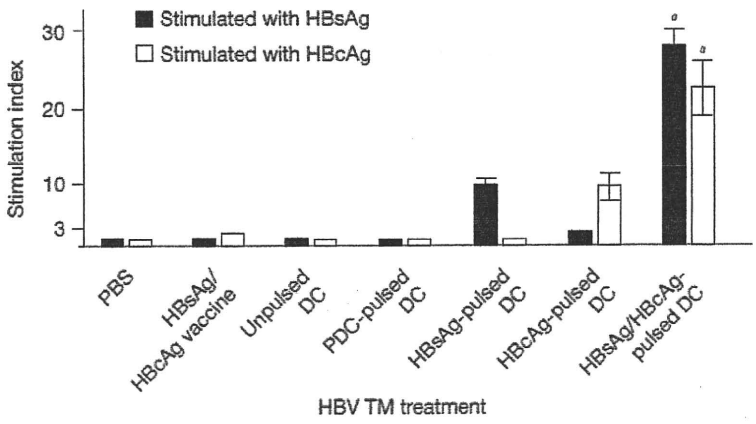
Normal C57BL/6J mice were immunized with hepatitis B surface antigen (HBsAg)/hepatitis B core antigen (HBcAg) or pyruvate dehydrogenase complex (PDC)-pulsed dendritic cells (DC), as described in *Methods*. Mice were euthanized 4 weeks after the second immunization, and spleen cells were injected with different stimulants. The levels of blastogenesis in cultures containing T-cells and irreverent antigen-pulsed DC were regarded as a stimulation index of 1.0. Data for stimulation indices are mean ±SD of three separate experiments.

Figure 2. Levels of HBsAg and anti-HBs in HBV TM before and after immunization with vaccines and antigen-pulsed DC



(A) HBV transgenic mice (TM) were immunized with different immunization regimens, as described in Figure 1. The levels of hepatitis B surface antigen (HBsAg) in the sera of different groups of mice before immunization and 4 weeks after second immunization are shown. (B) Antibody to HBsAg (anti-HBs) was not detected in HBV TM 4 weeks after injection with phosphate-buffered saline (PBS), vaccine containing HBsAg/hepatitis B core Antigen (HBcAg), unpulsed dendritic cells (DC), pyruvate dehydrogenase complex (PDC)-pulsed DC or HBcAg-pulsed DC. However, anti-HBs was detected in all HBV TM 1 month after immunization with HBsAg/HBcAg-pulsed DC. Data are mean ±SD of the levels of anti-HBs in sera. [a]$P<0.05$ compared with the levels of HBsAg before immunization. [b]$P<0.05$ compared with other groups.

Figure 3. Antigen-specific cellular immune responses caused by vaccination with HBsAg/HBcAg-pulsed DC



Hepatitis B surface antigen (HBsAg)- and hepatitis B core antigen (HBcAg)-specific proliferation was observed in T-cells from HBV transgenic mice (TM) immunized with HBsAg/HBcAg-pulsed dendritic cells (DC); however, proliferation did not result from immunization with other vaccine or DC formulations. Data are mean ±SD of levels of proliferation. [a]$P<0.05$ compared with other groups. PBS, phosphate-buffered saline; PDC, pyruvate dehydrogenase complex.

in the liver of HBV TM as a result of vaccination with HBsAg/HBcAg-pulsed DC. Liver NPC from HBV TM immunized with HBsAg/HBcAg-pulsed DC proliferated in response to stimulation with HBsAg (stimulation index 12.8 ±3.2; n=5) and HBcAg (stimulation index 16.4 ±4.5; n=5). However, liver NPC from HBV TM injected with PBS, HBsAg/HBcAg vaccine, unpulsed DC and PDC-pulsed DC did not proliferate in response to HBsAg or HBcAg (stimulation index <3.0). In addition, significantly higher numbers of IFN-γ-secreting $CD8^+$ T-cells were detected among liver NPC only from HBV TM immunized with HBsAg/HBcAg-pulsed DC, but not from other HBV TM (Figure 4). However, we did not observe an increase of alanine aminotransferase (before vaccination 29 ±4 IU/l versus 4 weeks after vaccination 27 ±3 IU/l) or evidence of liver damage in liver biopsy specimens in any HBV TM immunized with HBsAg/HBcAg-pulsed DC (SMFA *et al.*, data not shown).

Induction of antigen-specific lymphocytes from CHB patients by HBsAg/HBcAg-pulsed DC *in vitro*

To assess a clinical implication of this study regarding immunogenecity of HBsAg/HBcAg-pulsed DC in HBV TM, we performed an *in vitro* study using lymphocytes from patients with CHB. Autologous T-cells from patients with CHB were cultured with HBsAg/HBcAg-pulsed DC, unpulsed DC, PDC-pulsed DC or HBsAg/HBcAg vaccine. T-cells from CHB patients did not exhibit significant proliferation in response to unpulsed DC or PDC-pulsed DC. However, T-cells from CHB patients proliferated in the presence of HBsAg/HBcAg-pulsed autologous DC (Figure 5). Low levels of proliferation of autologous T-cells from one of five patients with CHB were also detected when these were cultured with HBsAg/HBcAg (Figure 5).

Antiviral capacity of HBsAg/HBcAg-pulsed DC in HBV TM

All HBV TM expressed HBsAg; however, free HBV DNA could be detected in some but not all HBV TM. To assess the antiviral capacity of HBsAg/HBcAg-pulsed DC, we immunized five HBV TM with detectable levels of HBV DNA using HBsAg/HBcAg-pulsed DC, unpulsed DC or PDC-pulsed DC. Levels of HBV DNA in the sera were decreased in all HBV TM as a result of immunization with HBsAg/HBcAg-pulsed DC. HBV TM expressed a mean ±SD level of 354 ±14 copies/ml of HBV DNA in the sera before vaccination. At 4 weeks after two vaccinations with HBsAg/HBcAg-pulsed DC, HBV DNA could not be detected in any HBV TM (level of detection; 200 copies/ml). However, no significant changes of HBV DNA levels were seen in HBV TM immunized with two injections of HBsAg/HBcAg vaccine, unpulsed DC or PDC-pulsed DC.

Discussion

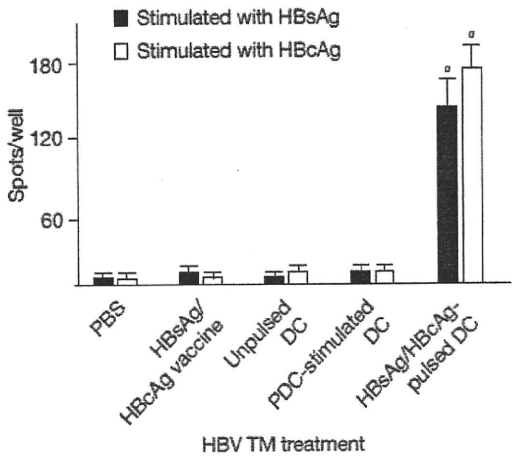
Several RCTs have documented the low therapeutic efficacy and considerable side effects of antiviral drugs used for CHB patients [2]. However, these drugs will remain the most useful and powerful tools for management of CHB patients [21] until more effective therapeutic regimens with fewer side effects can be developed. As an alternative therapeutic approach, polyclonal immune modulators have been used for

Table 2. IFN-γ-secreting T-cells in the spleen of HBV TM resulting from immunization with HBsAg/HBcAg-pulsed DC

HBV TM treatment	HBsAg-specific ELISPOT	HBcAg-specific ELISPOT
PBS	7 ±2	9 ±2
HBsAg/HBcAg vaccine	13 ±3	5 ±1
Unpulsed DC	11 ±4	12 ±3
PDC-pulsed DC	16 ±6	19 ±7
HBsAg/HBcAg-pulsed DC	198 ±23[a]	365 ±34[a]

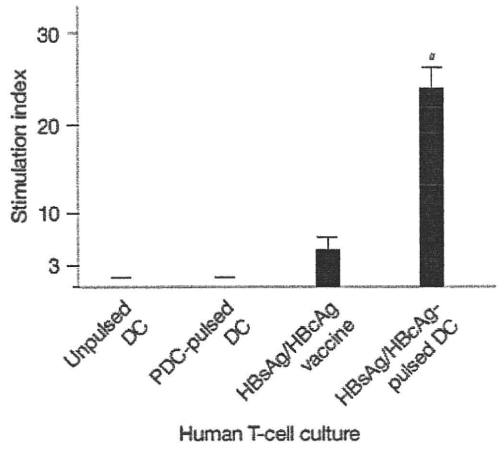
HBV transgenic mice (TM) were injected with phosphate-buffered saline (PBS) or immunized with hepatitis B surface antigen (HBsAg)/hepatitis B core antigen (HBcAg)-based vaccines, unpulsed dendritic cells (DC), pyruvate dehydrogenase complex (PDC)-pulsed DC or HBsAg/HBcAg-pulsed DC, twice at an interval of 4 weeks. HBV TM were euthanized 4 weeks after the second immunization, and T-cells were stimulated with HBsAg or HBcAg on an ELISPOT plate to assay the production of inteferon-γ spot. The spots were counted after deducting spots from control plates. Data are mean ±SD of three separate experiments. [a]P<0.05 compared with other groups.

Figure 4. IFN-γ-producing CTL in HBV TM immunized with HBsAg/HBcAg-pulsed DC



Significant numbers of interferon (IFN)-γ producing cytotoxic T-lymphocytes (CTL) were detected among liver non-parenchymal cells from HBV transgenic mice (TM) immunized with hepatitis B surface antigen (HBsAg)/hepatitis B core antigen (HBcAg)-pulsed dendritic cells (DC). Data are mean ±SE of four separate experiments. [a]P<0.05 compared to HBV TM immunized with other vaccine or DC formulations. PBS, phosphate-buffered saline; PDC, pyruvate dehydrogenase complex.

Figure 5. Antigen-specific lymphocytes from CHB patients by HBsAg/HBcAg-pulses DC *in vitro*



Antigen-specific proliferation of human peripheral blood T-cells from patients with chronic hepatitis B (CHB) resulting from stimulation with hepatitis B surface antigen (HBsAg)/hepatitis B core antigen (HBcAg)-pulsed dendritic cells (DC). The levels of blastogenesis in cultures containing T-cells and unpulsed DC were regarded as a stimulation index of 1.0. Data are mean and ±SD of five separate experiments. $^{a}P<0.05$ compared with HBV transgenic mice (TM) immunized with other vaccine or DC formulations. PDC, pyruvate dehydrogenase complex.

more than three decades in CHB patients; however, the clinical outcome with these agents has not been satisfactory [5]. To develop more effective immune therapy against HBV, vaccine therapy in which vaccines containing HBsAg were administered alone as well as in combination with other antiviral drugs in CHB patients [22,23]. Although some intermediate outcomes of CHB patients has been improved by this vaccine therapy, the present regimen of vaccine therapy still has limitations [6], as described in the *Introduction*.

The immune modulator effects of antigen-pulsed DC have been evaluated in HBV TM by other investigators, as well as our group [10,11]. Jiang *et al.* [10] used a murine model of HBV that expressed only HBsAg; the clinical implications of their study for translation research in patients with CHB are limited. We found that HBsAg-pulsed DC induced anti-HBs, but almost no HBsAg-specific cellular immunity, in HBV TM [11]. Also, we did not find therapeutic potentiality of HBsAg pulsed in CHB patients [24].

The present study has some noteworthy features. First, we used a vaccine that contains both HBsAg and HBcAg. We also prepared HBsAg/HBcAg-pulsed DC vaccine using a technique that we have been working with for more than a decade [14–16,19,20]. Next, an *in vitro* study revealed that HBsAg/HBcAg-pulsed DC could induce proliferation of both HBsAg- and HBcAg-specific lymphocytes from normal C57BL/6 mice. Subsequently, a preclinical trial using HBsAg/HBcAg-pulsed DC was accomplished in HBV TM. The data conclusively showed that HBsAg/HBcAg-pulsed DC induced anti-HBs in the sera, HBsAg and HBcAg-specific lymphocytes in the spleen, and HBsAg and HBcAg-specific CTLs in the spleen and the liver. Also, HBsAg/HBcAg-pulsed DC were able to reduce HBV DNA levels in HBV TM. Despite extensive immune modulating capacities of such preparations in HBV TM, we did not find any biochemical or histological evidence of liver injury with this approach, suggesting the viral clearance was mediated by non-cytotoxic effects of DC-based vaccine.

Our main target was to develop an effective immune therapeutic strategy for patients with CHB. Accordingly, we used a vaccine preparation that was a human-grade HBsAg and HBcAg. Indeed, clinical trials with this vaccine have already been performed in normal volunteers [18]. Accordingly, data from this study can be used to support the need for clinical trials using this vaccine. Further support of this concept was accumulated from data that showed that HBsAg/HBcAg-pulsed DC induced proliferation of both HBsAg- and HBcAg-specific T-cells from CHB patients (Figure 5).

The synergistic effect on the resulting immune response of HBsAg/HBcAg stimulation could be explained by the simultaneous stimulation of diverse Toll-like receptor (TLR) on DC. The nuclear content inside the *E.-coli*-derived recombinant HBcAg has been characterized as RNA (TLR3 and TLR7 ligands) [25–27]. Also, there is a recognized interaction between HBsAg and CD14, a component of TLR4 [28]. Finally, the aggregation of HBsAg and HBcAg in the liquid combined HBsAg/HBcAg formulation has been previously reported [25,29]. When these antigens were loaded on DC, antigen-pulsed DC were able to induce or activate HBsAg- and HBcAg-specific immune responses in HBV TM.

In conclusion, we have shown an improved immune therapeutic approach against chronic HBV infection. The antigens that we used are safe for human consumption and have been used in normal volunteers [18]. We prepared immunogenic antigen-pulsed DC with these antigens. HBsAg/HBcAg-pulsed DC induced both humoral and cellular immune responses in HBV TM *in vivo*; in addition, these DC induced proliferation of lymphocytes from CHB patients *in vitro*. We have already used HBsAg-pulsed DC in patients with CHB and confirmed its safety; however, only HBsAg-pulsed DC was not sufficiently effective to contain HBV replication and liver damages [24]. The next challenge will be to assess if HBsAg/HBcAg-pulsed DC can cause sustained control of HBV replication and reduction of liver damage in CHB, a finding that could not be completely assessed in HBV TM. The answer to that question will depend

on results of clinical trials in CHB patients, which are warranted based on the findings of this study.

Disclosure statement

The authors declare no competing interests.

References

1. Férir G, Kaptein S, Neyts J, De Clercq E. Antiviral treatment of chronic hepatitis B virus infections; the past, the present and the future. *Rev Med Virol* 2008; 18:19–34.
2. Shamliyan TA, MacDonald R, Shaukat A, *et al.* Antiviral therapy for adults with chronic hepatitis B: a systemic review for a National Institutes of Health Consensus Development Conference. *Ann Intern Med* 2009; 150:111–124.
3. Rehermann B. Chronic infections with hepatotropic viruses: mechanisms of impairment of cellular immune responses. *Semin Liver Dis* 2007; 27:152–160.
4. Boni C, Penna A, Ogg GS, *et al.* Lamivudine treatment can overcome cytotoxic T-cell hyporesponsiveness in chronic hepatitis B: new perspectives for immune therapy. *Hepatology* 2001; 33:963–971.
5. Sprengers D, Janssen HL. Immunomodulatory therapy for chronic hepatitis B virus infection. *Fundam Clin Pharmacol* 2005; 19:17–26.
6. Pol S, Michel ML. Therapeutic vaccination in chronic hepatitis B virus carriers. *Expert Rev Vaccines* 2006; 5:707–716.
7. Onji M, Akbar SM. *Dendritic Cell in Clinics.* 2nd ed. Tokyo: Springer 2008.
8. van der Molen RG, Sprengers D, Binda RS, *et al.* Functional impairment of myeloid and plasmacytoid dendritic cells of patients with chronic hepatitis B. *Hepatology* 2004; 40:738–746.
9. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. *Annu Rev Immunol* 1995; 13:29–60.
10. Jiang WZ, Fan Y, Liu X, *et al.* Therapeutic potential of dendritic cell-based immunization against HBV transgenic mice. *Antiviral Res* 2008; 77:50–55.
11. Akbar SM, Furukawa S, Hasebe A, Horiike N, Michitaka K, Onji M. Production and efficacy of a dendritic cell-based therapeutic vaccine for murine chronic hepatitis B virus carrierer. *Int J Mol Med* 2004; 14:295–299.
12. Araki K, Miyazaki J, Hino O, *et al.* Expression and replication of hepatitis B virus genome in transgenic mice. *Proc Natl Acad Sci U S A* 1989; 86:207–211.
13. Niiya T, Akbar SM, Yoshida O, *et al.* Impaired dendritic cell function resulting from chronic undernutrition disrupts the antigen-specific immune response in mice. *J Nutr* 2007; 137:671–675.
14. Akbar SM, Onji M, Inaba K, Yamamura K, Ohta Y. Low responsiveness of hepatitis B virus-transgenic mice in antibody response to T-cell-dependent antigen: defect in antigen-presenting activity of dendritic cells. *Immunology* 1993; 78:468–475.
15. Yoshida O, Akbar F, Miyake T, *et al.* Impaired dendritic cell functions because of depletion of natural killer cells disrupt antigen-specific immune responses in mice: restoration of adaptive immunity in natural killer-depleted mice by antigen-pulsed dendritic cell. *Clin Exp Immunol* 2008; 152:174–181.
16. Akbar SM, Furukawa S, Yoshida O, Hiasa Y, Horiike N, Onji M. Induction of anti-HBs in HB vaccine nonresponders *in vivo* by hepatitis B surface antigen-pulsed blood dendritic cells. *J Hepatol* 2007; 47:60–66.
17. Akbar SM, Furukawa S, Nakanishi S, Abe M, Horiike N, Onji M. Therapeutic efficacy and decreased nitrite production by bezafibrate in patients with primary biliary cirrhosis. *J Gastroenterol* 2005; 40:157–163.
18. Betancourt AA, Delgado CA, Estévez ZC, *et al.* Phase I clinical trial in healthy adults of a nasal vaccine candidate containing recombinant hepatitis B surface and core antigens. *Int J Infect Dis* 2007; 11:394–401.
19. Akbar SM, Abe M, Masumoto T, Horiike N, Onji M. Mechanism of action of vaccine therapy in murine hepatitis B virus carriers: vaccine-induced activation of antigen presenting dendritic cells. *J Hepatol* 1999; 30:755–764.
20. Hasebe A, Akbar SM, Furukawa S, Horiike N, Onji M. Impaired functional capacities of liver dendritic cells from murine hepatitis B virus (HBV) carriers: relevance to low HBV-specific immune responses. *Clin Exp Immunol* 2005; 139:35–42.
21. Keeffe EB, Dieterich DT, Han SH, *et al.* A treatment algorithm for the management of chronic hepatitis B virus infection in the United States: 2008 update. *Clin Gastroenterol Hepatol* 2008; 6:1315–1341.
22. Pol S. Immunotherapy of chronic hepatitis B by anti HBV vaccine. *Biomed Pharmacother* 1995; 49:105–109.
23. Horiike N, Akbar SM, Michitaka K, *et al. In vivo* immunization by vaccine therapy following virus suppression by lamivudine: a novel approach for treating patients with chronic hepatitis B. *J Clin Virol* 2005; 32:156–161.
24. Akbar SM, Yoshida O, Furukawa S, Hiasa Y, Horiike N, Onji M. Induction of antigen-specific humoral and cellular immune responses by antigen-pulsed dendritic cells in hepatitis B vaccine nonresponders and patients with chronic hepatitis B. *58th Annual Meeting of the American Association for the Study of Liver Diseases.* 2–6 November 2007, Boston, MA, USA. Abstract 110.
25. Lobaina Y, Palenzuela D, Pichardo D, Muzio V, Guillén G, Aguilar JC. Immunological characterization of two hepatitis B core antigen variants and their immunoenhancing effect on co-delivered hepatitis B surface antigen. *Mol Immunol* 2005; 42:289–294.
26. Vanlandschoot P, Cao T, Leroux-Roels G. The nucleocapsid of the hepatitis B virus: a remarkable immunogenic structure. *Antiviral Res* 2003; 60:67–74.
27. Lee BO, Tucker A, Frelin L, *et al.* Interaction of the hepatitis B core antigen and the innate immune system. *J Immunol* 2009; 182:6670–6681.
28. Vanlandschoot P, Van Houtte F, Hoek F, Nieuwland R, Leroux-Roels G. Saccharomyces cerevisiae-derived HBsAg preparations differ in their attachment to monocytes, immune-suppressive potential, and T-cell immunogenicity. *J Med Virol* 2003; 70:513–519.
29. Aguilar JC, Lobaina Y, Muzio V, *et al.* Development of a nasal vaccine for chronic hepatitis B infection that uses the ability of hepatitis B core antigen to stimulate a strong Th1 response against hepatitis B surface antigen. *Immunol Cell Biol* 2004; 82:539–546.

Accepted for publication 25 March 2010

Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/j.1440-1746.2009.06037.x

HEPATOLOGY

Radiofrequency ablation therapy for hepatocellular carcinoma in elderly patients

Atsushi Hiraoka,* Kojiro Michitaka,* Norio Horiike,* Satoshi Hidaka,* Takahide Uehara,* Soichi Ichikawa,* Aki Hasebe,* Yasunao Miyamoto,* Tomoyuki Ninomiya,* Ichiro Sogabe,† Yoshihiro Ishimaru,† Hideki Kawasaki,‡ Yohei Koizumi,§ Masashi Hirooka,§ Yoshimasa Yamashita,§ Masanori Abe,§ Yoichi Hiasa,§ Bunzo Matsuura§ and Morikazu Onji§

Departments of *Gastroenterology, †Radiology, and ‡Surgery, Ehime Prefectural Central Hospital, and §Gastroenterology and Metabology, Ehime University Graduate School of Medicine, Ehime, Japan

Key words
elderly, hepatocellular carcinoma, radiofrequency ablation.

Accepted for publication 22 July 2009.

Correspondence
Dr Atsushi Hiraoka, Ehime Prefectural Central Hospital, Kasuga-Cho 83, Matsuyama, Ehime 790-0024, Japan. E-mail: hirage@m.ehime-u.ac.jp

Abstract

Background and Aim: With the aging of society, the number of elderly patients with hepatocellular carcinoma (HCC) has been increasing in Japan. The Government of Japan defines elderly as being over 65 and has divided the elderly into two stages: the first elderly stage (< 75 years old) and the second elderly stage (≥ 75). We investigated the efficacy and safety of radiofrequency ablation therapy (RFA) in patients in the second elderly stage in comparison with other HCC patients, retrospectively.

Methods: Two hundred six patients with HCC, who were within the Milan criteria, with low-grade performance status (0 or 1) and a Child-Pugh classification of A or B were enrolled. All were treated with RFA from January 2000 to December 2008 as an initial therapy and were divided into elderly HCC group (e-HCC group; ≥ 75, $n = 63$) and non e-HCC group (< 75, $n = 143$), and their clinical data and survival rates were compared.

Results: Age and the level of protein induced by vitamin K absence or antagonist (PIVKA-II) were higher in the e-HCC group as compared with the non e-HCC group (78.3 ± 3.2 *vs* 64.2 ± 7.5 years, 676.3 ± 2643.7 *vs* 142.4 ± 442.2 mAU/mL: $P < 0.01$, respectively). There were no significant differences for Child-Pugh class, tumor node metastasis stage, and Japan Integrated Stage score and in survival rates after 3, and 5 years between the groups (e-HCC group: 82.5% and 49.7%, respectively; non e-HCC group: 78.3% and 57.5%, respectively). There were no severe complications in the e-HCC group.

Conclusions: Elderly HCC patients, who have good performance status, should be treated in the same manner and with the same strategy as young HCC patients.

Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related death worldwide and is rapidly increasing.[1–3] With the aging of society in Japan, the number of elderly patients with HCC has been increasing.[4]

Various therapeutic modalities including liver transplantation,[5] surgical resection,[6,7] percutaneous ethanol injection therapy,[8] radiofrequency ablation therapy (RFA),[9] and transcatheter arterial chemoembolization (TACE)[10,11] are used. In many patients with HCC, liver cirrhosis with low grade Child-Pugh classification[12] due to hepatitis virus is often present. Poor hepatic reserve function makes it difficult to carry out surgical resection. Liver transplantation is limited for HCC patients because of the lack of donors in Japan. Recently, RFA has become widely used throughout the world, mainly because of its ease of use, lower level of invasiveness, and effectiveness.[13,14] Though elderly patients with HCC have been increasing, conclusive evidence showing that RFA is safe and effective for elderly HCC patients has not been reported, to the best of our knowledge. The Ministry of Health, Labour and Welfare, Government of Japan defines elderly as being over 65 and has further divided the term into two stages: the first elderly stage (< 75 years old) and the second elderly stage (≥ 75). In the present study, we investigated the efficacy and safety of RFA in HCC patients of the second elderly stage (≥ 75) in comparison with those in HCC patients less than 75 years old.

Methods

Seven hundred and twenty-seven patients were diagnosed as having HCC and were treated at Ehime Prefectural Central Hospital from January 2000 to December 2008. We retrospectively analyzed clinical data, prognosis and the frequency of complications for 206 HCC patients, who were treated with RFA, within the Milan criteria

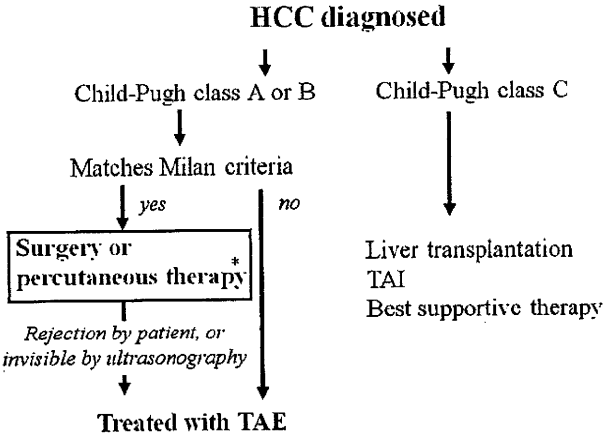


Figure 1 Hepatocellular carcinoma (HCC) treatment strategy at Ehime Prefectural Central Hospital. *Surgical resection is considered the first option in the cases that have good hepatic reserve function, and this is explained to the patient. When patients wish to be treated with radiofrequency (RFA) or have some reasons for refusing surgical resection, RFA is selected. TAE, transcatheter arterial embolization (chemoembolization); TAI, transcatheter arterial infusion chemotherapy.

(single lesion ≤ 5 cm or two to three lesions each ≤ 3 cm),[5] with low-grade performance status (PS: 0 or 1) and a Child-Pugh classification of A or B. All subjects had no past history of treatment for HCC. We divided the patients into elderly (≥ 75 years old, e-HCC group, $n = 63$) and non-elderly (< 75 years old, non-e-HCC group, $n = 143$), and compared their clinical data and survival rates.

The diagnosis of HCC was based on pathological findings, or imaging evidence of tumor formation in the liver (with arterial hypervascularization) on at least two imaging modalities (dynamic computed tomography [CT],[15] ultrasonography [US], contrast enhanced ultrasonography [CEUS][16] and angiography).

The strategy for HCC treatment at our institution is shown in Figure 1, with surgical resection considered to be the first option in the cases that have good hepatic reserve function, and which is explained to each patient. When patients wish to be treated with RFA or have reasons for refusing surgical resection, RFA is selected. In addition, all recent cases were treated with reference to the guidelines of Japan for HCC.[17] Informed consent was received from all of the patients in the present study. Prior to percutaneus RFA treatment, 15 mg of Pentazocine hydrochloride and 25 mg of hydroxyzine hydrochloride were given intramuscularly. Local anesthesia was induced by 5 mL of 1% Lidocaine injected through the skin into the peritoneum along a predetermined puncture line. In cases in which the tumor was difficult to visualize using conventional US, we used CEUS[18] with Perflubutane (Sonazoid, Daiichi-Sankyo Co. Ltd. Tokyo, Japan)[16,19] and/or artificial pleural effusion and ascites[20] as assistant methods for RFA. Midazolam (Dormicum, Astellas Pharma Inc., Tokyo, Japan) was injected intravenously at the start of ablation (0.1 mg/kg). Hypervascular HCC were subjected to TACE[10,11] with Epirubicin-Lipiodol emulsion and gelatin sponge (Gelfoam, Upjohn, Kalamazoo, MI, USA) or multiporous gelatin particles (Gelpart, Astellas Pharma Inc. Japan) 3–5 days before RFA. RFA was carried out with sonographic guidance (Aloka 5500, Aloka α-10 [Aloka Co., Ltd, Tokyo, Japan] or EUB7500 [Hitachi Medical corp., Tokyo, Japan]) using a convex or microconvex probe. In difficult cases for percutaneous insertion, we tried laparoscopic and thoracoscopic RFA procedures. We inserted a 20-cm long 17-gauge radiofrequency electrode equipped with a 2- or 3-cm long exposed metallic tip (Radionics Cool-tip, Burlington, MA, USA).

Dynamic CT was carried out for all nodules at 3–5 days after RFA procedures. The necrotic area of the liver parenchyma surrounding the target lesion was revealed as hypoattenuating during the portal phase of dynamic CT. When a necrotic area larger than the target tumor was obtained in post-RFA dynamic CT as compared with pre-RFA dynamic CT, the procedure was judged to be successful[21] by two expert radiologists and/or hepatologists.

Statistical analysis

The data are expressed as the mean ± standard deviation (SD). Statistical analyses were carried out using student's *t*-test for unpaired data, χ^2 square test, Fischer's exact test and a Mann–Whitney *U*-test as appropriate. All statistical analyses were carried out by SPSS 16.0J (SPSS Japan Inc., Tokyo, Japan). A *P*-value of less than 0.05 was considered to represent statistical significance.

Results

None of the patients had a past history of HCC. Age and the level of protein induced by vitamin K absence or antagonist (PIVKA-II) were higher in the e-HCC group as compared with the non-e-HCC group (78.3 ± 3.2 [range: 75–93] *vs* 64.2 ± 7.5 [range: 38–74] years, 676.3 ± 2643.7 *vs* 142.4 ± 442.2 mAU/mL: $P < 0.01$, respectively) (Table 1). In contrast, there were no significant differences for Child-Pugh classification, tumor node metastasis (TNM) stage,[22] and Japan Integrated Stage (JIS) score[23] between the groups. There were also no significant differences in survival rates after 1, 3, and 5 years (e-HCC group: 91.7%, 82.5%, and 49.7%, respectively; non-e-HCC group: 93.2%, 78.3%, 57.5%, respectively) (Fig. 2). Disease-free survival rate is shown in Figure 3; there was no significant difference between both groups. In the e-HCC group, there was one case of biloma and two of dermatitis combustionis observed as complications, while there were two cases of hemothorax, one of pleurisy, one of ecchymoma, two of dermatitis combustionis, and three of inflammation of the organs near the tumor in the non-e-HCC group ($P = 0.913$). The values for frequency of synchronous other malignancy, which were controlled well with curative therapies, heterocronous other malignancy, and mortality from extrahepatic disease were not significantly different between the groups (e-HCC group *vs* non-e-HCC group: 3.2% [2/63] *vs* 4.9% [7/143]; $P = 0.725$, 3.2% [2/63] *vs* 6.3% [9/143]; $P = 0.509$, and 38.5% [5/13] *vs* 23.7% [9/38]; $P = 0.309$, respectively) (Table 2). In both groups, no reduction in Child-Pugh score was observed at the first instance of recurrence.

Discussion

With aging societies, increasing elderly HCC patients has become a worldwide trend. Though, liver transplantation, surgical resection, percutaneous ethanol injection therapy (PEIT), RFA and