Feasibility of Auxiliary Partial Orthotopic Liver Transplantation from Living Donors for Patients with Adult-Onset Type II Citrullinemia

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More than 20 patients with adult-onset type II citrullinemia have undergone liver transplantation, showing dramatic therapeutic effects. In Japan, living donor liver transplantation is the standard technique of liver transplantation because of the rare availability of cadaveric donors. The feasibility of auxiliary partial orthotopic liver transplantation (APOLT) for adult-onset type II citrullinemia to overcome the problem of a small-for-size graft in living donor liver transplantation has not been defined. We recently performed APOLT for patients with type II citrullinemia. Here, we present 2 patients: patient 1 was a 32-year-old man and patient 2 was a 43-year-old woman. Both patients suffered from hepatic encephalopathy, and laboratory data showed highly elevated plasma levels of ammonia and citrulline. In patient 1, the liver graft was obtained from a patient with familial amyloid polyneuropathy as a domino liver transplant. In patient 2, APOLT was performed after graft donation from her husband. The postoperative clinical courses of both patients were uneventful, and the neurological symptoms were completely resolved. The plasma concentrations of ammonia and citrulline normalized rapidly in both patients. APOLT can provide an adequate hepatocyte mass to correct the underlying enzyme deficiency in adult patients with type II citrullinemia. In addition, APOLT can be carried out safely to overcome the limitation of graft volume in living donor liver transplantation. (*Liver Transpl 2004;10:550–554.*)

Citrullinemia is a rare hereditary metabolic disorder characterized by highly elevated plasma levels of citrulline and ammonia, and is ascribed to a deficiency of argininosuccinate synthetase in the liver.[1] This disorder can be classified into 3 types: neonatal/infantile (types I and III) and adult (type II).[2] Most of the patients with type II citrullinemia have been reported in Japan, and the causative gene of this disorder, citrin, was recently identified.[3,4] In the past, most patients were treated with medication and died of severe brain edema within a few years after disease onset, but during the last decade a small number of patients have undergone liver transplantation,[5–8] showing dramatic therapeutic effects. In Japan, since the cadaveric donor pool is quite limited, living donor liver transplantation has become the standard alternative technique.[8] However, living donor liver transplantation requires a candidate living donor among the patient's family with sufficient liver graft volume.

We report the cases of 2 patients with type II citrullinemia who were treated successfully with auxiliary partial orthotopic liver transplantation (APOLT). This procedure was selected based on preoperative volumetric analysis of the liver graft, which did not reach 40% of the recipient's standard liver volume. The first patient underwent APOLT using a graft obtained from a patient with familial amyloid polyneuropathy (FAP) as a domino liver transplant. This is the first description of APOLT for patients with type II citrullinemia.

Case Reports

Patient 1

This patient was a 32-year-old Japanese man who had been found to have mild liver dysfunction at age 22, when a liver biopsy revealed steatotis. On January 17, 2003, he suddenly suffered severe consciousness disturbance after drinking a small amount of alcohol. Subsequently, he became irritable

Abbreviations: APOLT, auxiliary partial orthotopic liver transplantation; FAP, familial amyloid polyneuropathy; ARG, arginase; ASL, argininosuccinate lyase; ASS, argininosuccinate synthetase; CPS, carbamyl phosphate synthetase; OTC, ornithine transcarbamylase.

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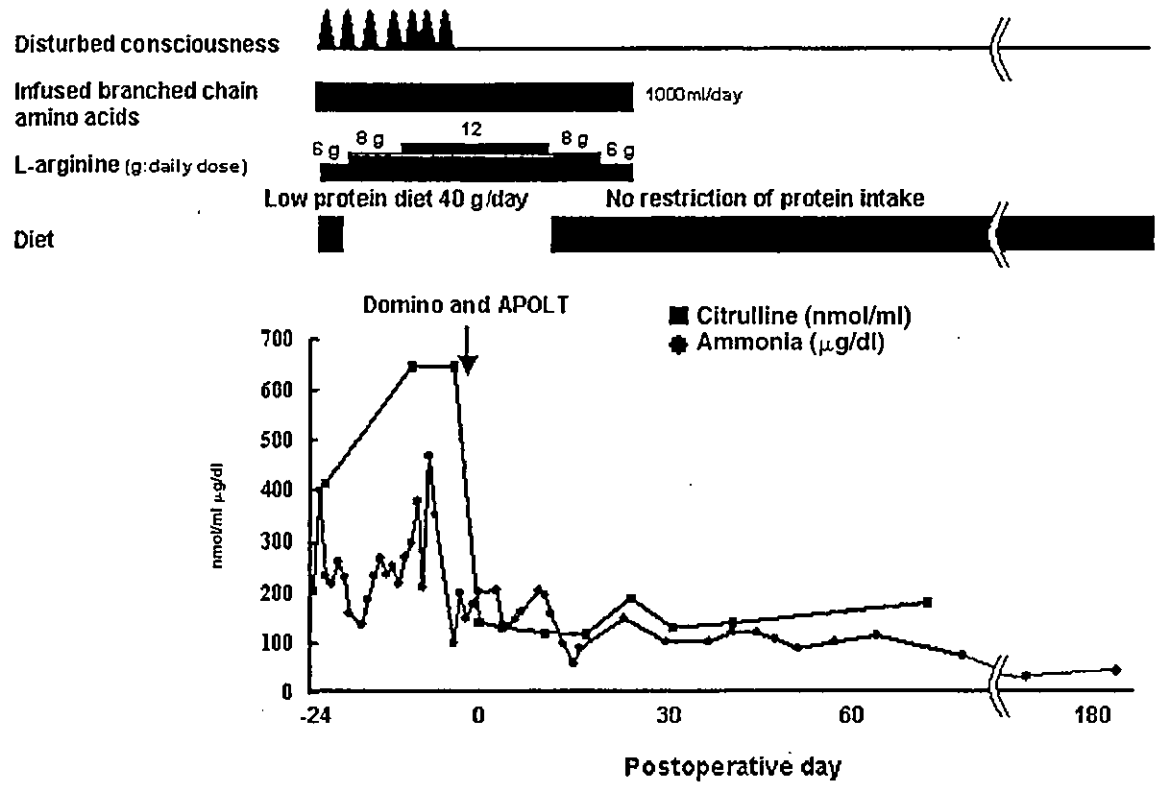

Figure 1. Clinical course and serial changes in plasma citrulline and ammonia levels before and after liver transplantation in patient 1.

and began to verbally abuse his family. He also showed disorientation and became very forgetful. He was admitted to a local hospital on January 23, and a laboratory examination revealed a raised plasma level of ammonia. He was referred to our hospital for further examination on January 25.

The patient was 170 cm in height and 52 kg in weight. He was highly irritable and confused, and showed flapping tremor. His plasma levels of ammonia and citrulline were highly elevated to 192 μg/dl (normal < 80 μg/dl) and 416.4 nmol/ml (normal < 60 nmol/ml), respectively. The plasma arginine concentration was mildly elevated to 147.7 nmol/ml (normal < 130 nmol/ml). Computed tomography showed a diffuse low-density area in the liver, suggesting severe steatosis. An electroencephalogram showed diffuse slow waves including frequent discharge of triphasic waves. DNA analysis of the citrin gene revealed that the patient was homozygous for the Ser225X mutation,[3] and, therefore, he was diagnosed as having type II citrullinemia. A low protein diet (protein 40 g/day) and oral administration of lactulose, kanamycin (1500 mg/day) and L-arginine (12 g/day)[9] were started. Subsequently, intravenous hyperalimentation with branched chain amino acids was also begun instead of the diet. Despite these treatments, his consciousness disturbance with disorientation and abnormal behavior did not improve and his plasma concentrations of ammonia and citrulline continued to increase (Fig. 1). Consequently, urgent liver transplantation seemed the only option. Although we considered living donor liver transplantation, unfortunately, there was no appropriate donor candidate in his family. Moreover, cadaveric liver transplantation seemed improbable because of the serious shortage of cadaveric donors in Japan. Therefore, we finally planned to perform combined domino transplantation and APOLT using a graft from a 52-year-old female patient with FAP (a Val30Leu transthyretin gene mutation), for whom living donor liver transplantation had already been planned. Informed consent was obtained from both the patient with type II citrullinemia (recipient) and the patient with FAP (domino transplant donor), and liver transplantation was performed on February 18, 2003, after approval by the local ethics committee. The recipient's left hepatic lobe (segments 1–4 according to Couinaud's segmentation) was resected and the domino left liver graft (segments 2–4) was transplanted orthotopically. The right hepatic lobe of the recipient was not removed and the right anterior portal vein was ligated and transected, leaving the right posterior portal vein patent. This procedure causes hypertrophy of the left liver graft as well as atrophy of the recipient's right anterior segment. The domino left liver weighed 284 g, corresponding to only 26% of the recipient's estimated whole liver volume. The details of this surgical procedure have been reported elsewhere.[10–12] Histo-

Table 1. Results of Urea Cycle Enzyme Analysis (Unit/mg Protein) of the Resected Liver

	CPS	OTC	ASS	ASL	ARG
Patient 1	0.023	0.94	0.0055	0.049	6.6
Patient 2	0.08	1.97	0.007	0.071	11.9
Controls	0.036 ± 0.013	0.88 ± 0.35	0.033 ± 0.012	0.052 ± 0.025	15.8 ± 3.1

Abbreviations: CPS, carbamyl phosphate synthetase; OTC, ornithine transcarbamylase; ASS, arginosuccinate synthetase; ASL, arginosuccinate lyase; ARG, arginase.

logical examination of the resected left lobe revealed severe fatty change. Five urea cycle enzyme analyses of the resected liver revealed that only argininosuccinate synthetase activity was extremely low (Table 1). After transplantation, the patient began receiving routine immunosuppression therapy. All of his neurological symptoms including the consciousness disturbance and flapping tremor disappeared within 3 days after transplantation. The plasma concentrations of citrulline and ammonia declined rapidly without either any specific medication or nutritional support for hyperammonemia (Fig. 1). Electroencephalography on postoperative days 10 and 50 showed complete disappearance of abnormal waves. The postoperative course of this patient was uneventful. Computed tomographic volumetry of the graft on postoperative day 28 gave value of 409 ml. Postoperative analysis of serum transthyretin in the serum of patient 1 using matrix-assisted laser desorption ionization and time-of-flight mass spectrometry[13] revealed both wild-type transthyretin (Val30) and variant transthyretin (Leu30), the latter produced by the liver graft donated from the FAP patient (Fig. 2).

Patient 2

The patient was a 43-year-old Japanese woman who had started to experience occasional headaches, drowsiness, and abnormal behavior at age 40. She was admitted to a hospital because of consciousness disturbance at age 42. Laboratory examination revealed hyperammonemia (583 μg/dl) and hypercitrullinemia (214.9 nmol/ml). DNA analysis of the citrin gene revealed that the patient was homozygous for the Ser225X mutation.[3] She was, therefore, diagnosed as having type II citrullinemia, and oral administration of L-arginine (9 g/day) and sodium benzoate (9 g/day) was started. However, her plasma ammonia level continued to increase (866 μg/dl), necessitating referral to our hospital on January 27, 2003. At this time, her consciousness was clear and fine postural tremor of both hands was seen. Her plasma ammonia and citrulline concentrations were 201 μg/dl and 605.8 nmol/ml, respectively. Abdominal computed tomography demonstrated a diffuse low-density area in the liver, and electroencephalography showed frequent slow waves with triphasic waves. She was treated by low protein diet (30 g/day) and oral administration of L-arginine (16 g/day), sodium benzoate (9 g/day), kanamycin (1500 mg/day), and branched amino acids. However, her plasma ammonia level did not decrease, and occasional drowsiness was seen (Fig. 3). On April 6, her plasma ammonia level became highly increased, and intravenous hyperalimentation with branched chain amino acids was begun after withdrawal of the oral diet. These treatments failed to improve her condition, and, therefore, she underwent partial liver trans-

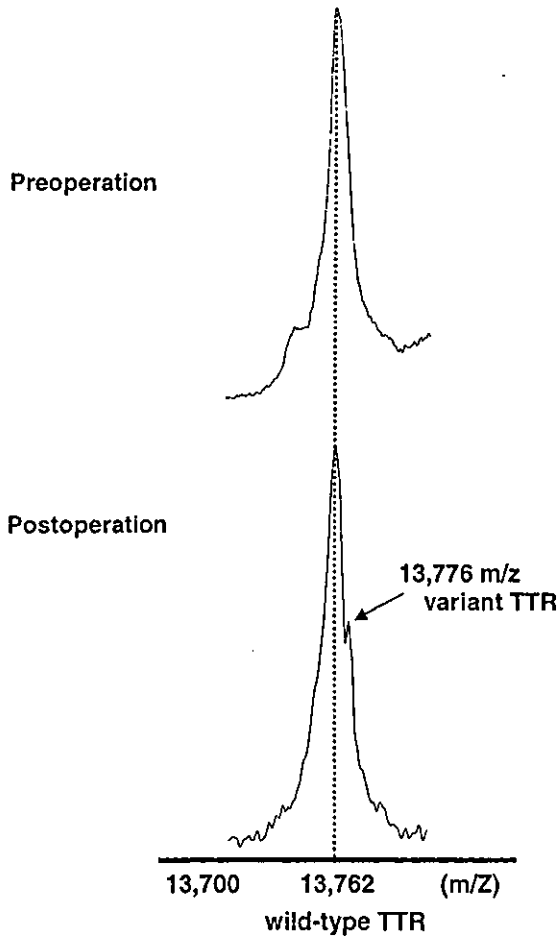

Figure 2. Mass spectra of immunoprecipitated serum transthyretin in patient 1, obtained by matrix-assisted laser desorption ionization and time-of-flight mass spectrometry.[13] Wild-type transthyretin (Val30) is identified as an ion peak of 13,762 m/z and the variant transthyretin (Leu30) is 14 m/Z larger than the wild-type transthyretin.

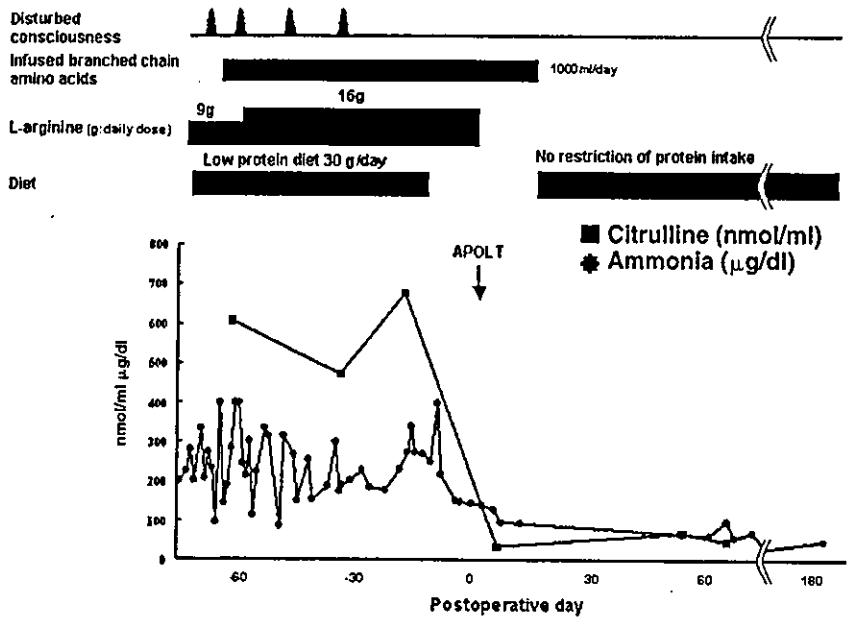

Figure 3. Clinical course and serial changes in plasma citrulline and ammonia levels before and after liver transplantation in patient 2.

plantation on April 15, 2003, using a left lobe graft from her husband. Since the graft volume obtained from her husband was insufficient (384 g), corresponding to 39.1% of the patient's standard liver volume (981 ml), we eventually decided to perform APOLT. The patient's left lobe (segments 1–4) was resected leaving her right hepatic lobe intact. The left liver graft (segments 2–4) was transplanted orthotopically and the recipient's right portal vein was ligated and transected. The postoperative course was uneventful, and the neurological symptoms were resolved on postoperative day 2. Histological examination of the patient's left lobe revealed severe steatosis. Urea cycle enzyme analysis of the liver demonstrated argininosuccinate synthetase deficiency (Table 1). The plasma concentrations of citrulline and ammonia normalized rapidly without any specific medication or nutritional support (Fig. 3). The computed tomography estimated graft volume on postoperative day 28 was 769 ml.

Discussion

We have described 2 adult patients with type II citrullinemia who underwent APOLT successfully with small left liver grafts from living donors.

The preoperative clinical condition of patient 1, including laboratory data, deteriorated rapidly despite several courses of intensive treatment for hepatic encephalopathy, and preoperative computed tomography volumetry showed that the left lobe of the FAP patient was small for use as a graft. We, therefore, decided to perform combined domino transplantation and APOLT for this patient. In preparing this procedure for patient 1, it was an issue of repeated discussion whether to divide the recipient's right portal vein completely or partially. We elected to divide his right anterior portal venous branch for the following 2 reasons. First, the possible development of FAP in patient 1 in the future (probably within 20 to 30 years after transplantation) would jeopardize his life. Considering the rapid advances in medical treatment for metabolic disorders including FAP and citrullinemia, division of his right portal venous trunk would eliminate the possibility of removing the transplanted left liver if gene treatment for citrullinemia were ever established in the future. Second, because the estimated graft volume was too small for patient 1 (corresponding to 26% of the recipient's standard liver volume), it was considered beneficial to leave the right posterior portal vein patent to avoid exposing the graft to portal venous hypertension, which is assumed to be an adverse factor in liver transplantation with a small-for-size graft.

In patient 2, we considered that the graft volume (384 g: 39.1% of the recipient's standard liver volume) was not sufficient for the recipient, and, therefore, decided to perform APOLT for her. In contrast to patient 1, however, we divided the right portal venous trunk because the graft volume was not too small and there would be little likelihood of needing to extirpate the transplanted normal liver graft.

APOLT was initially introduced as a temporary or

permanent support for patients with fulminant hepatic failure,[14] and its indications have been extended to liver-based metabolic disorders. At our institution, APOLT has been carried out for patients with FAP to guarantee a sufficient margin of safety for both donor and recipient when a living donor's left lobe volume is insufficient for the recipient.[10]

With regard to urea cycle disorders, APOLT has been performed for a few patients with ornithine transcarbamylase deficiency.[15] Kasahara et al.[15] proposed that, for such patients, removal of the whole native liver is unnecessary for 3 reasons: a partial liver segment with normal enzyme activity corrects the hyperammonemia; if graft failure occurs, the remnant native liver is an available backup; and the remnant liver could benefit in the future from potential advances in gene treatment. In both patients 1 and 2, we also decided not to remove the remnant native liver later, as mentioned by Kasahara et al.[15] In particular, although in patient 1 the graft volume was only 26% of the estimated standard liver volume, transplantation dramatically improved his systemic condition and laboratory, data as shown in Fig. 1. These results indicate that APOLT can provide an adequate hepatocyte mass to correct the underlying metabolic abnormality in adult patients with type II citrullinemia. Meanwhile, because around 8% of patients with type II citrullinemia are reported to develop hepatocellular carcinoma,[16] careful follow-up is mandatory.

In conclusion, APOLT can be a safe and effective treatment for patients with type II citrullinemia, especially those who have candidate donors with insufficient graft volume or who have a chance of receiving a domino liver graft.

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ELSEVIER

Conversion from Tacrolimus to Cyclosporine Microemulsion Therapy in Liver Transplant Recipients

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ABSTRACT

The calcineurin inhibitors cyclosporine and tacrolimus have distinct advantages and drawbacks. Therefore it is important to tailor their use to the patient's tolerance. In some patients, the need to ameliorate the adverse effects of tacrolimus may necessitate a switch to cyclosporine-based therapy. Rescue therapy with a cyclosporine microemulsion (Neoral)-based regimen for transplant patients intolerant of tacrolimus has been evaluated to assess the best method of switching and determine the initial and maintenance doses of Neoral in children and adults. Our aims were to evaluate not only these facets, but also the pharmacokinetics of Neoral in stable patients, including target 2-hour postdose blood concentrations (C_2) of cyclosporine in liver transplant recipients. Eighteen liver transplant patients switched from tacrolimus to Neoral underwent a program of cyclosporine blood level monitoring. The conversions were conducted safely; the incidence of acute rejection episodes was low (11.1%). Statistical analysis showed that the C_2 correlated with the area under the time-blood concentration curve of cyclosporine for 0 to 4 hours after dosing ($R = 0.970$). We determined the maintenance doses of Neoral for pediatric and adult patients as well as the feasibility of C_2 quantitated monitoring in liver transplantation.

THE CALCINEURIN INHIBITORS cyclosporine and tacrolimus represent the mainstays of current immunosuppressive regimens for the prevention of acute rejection after liver transplantation.[1-3] Because these drugs have their own advantages and drawbacks,[4,5] it is important to tailor their use to patient tolerance. From our experience in living donor liver transplantation, the incidence of acute cellular rejection is lower using tacrolimus- than cyclosporine-based regimens.[6] However, the adverse effects of tacrolimus have affected its clinical utility in some patients.[3-5] The need to ameliorate tacrolimus-associated adverse effects, such as gastrointestinal intolerance, neurotoxicity, and diabetes mellitus, may prompt a switch to cyclosporine-based therapy.[7] The validity of rescue therapy with a cyclosporine microemulsion (Neoral)-based regimen in transplant patients intolerant of tacrolimus has been evaluated in several centers.[7] Recently, Abouljoud et al[8] reported positive results from a multicenter, open-label, single-arm prospective cohort study. There is now a need to investigate the best method and any contraindications to switch patients from tacrolimus to Neoral, as well as to determine the initial and maintenance doses of Neoral in children and adults. We not only evaluated these issues, but also the pharmacokinetics of Neoral in stable patients, especially estimating target 2-hour postdose blood concentrations (C_2) of cyclosporine in liver transplant recipients.

PATIENTS AND METHODS

Between September 1993 and August 2003, 204 patients (mean age 23.5 ± 22.6 years) underwent primary liver transplantation under tacrolimus-based immunosuppression. Among them, 18 patients (aged 8 months to 61 years; 11 male, 7 female) were switched from tacrolimus to Neoral (at 18 to 2610 days after liver transplantation) and underwent monitoring of cyclosporine blood levels. Nine of the 18 subjects were 15 years or younger. The conditions necessitating

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Table 1. Mean Blood Concentrations of Cyclosporine After Neoral Administration in Living Donor Liver Transplant Recipients

	2–6 Months After Liver Transplantation				More Than 6 Months After Liver Transplantation			
		C_0	C_2	AUC_{0-4}		C_0	C_2	AUC_{0-4}
Ä	Dose of Neoral (mg/kg per day)	(ng/mL)			Dose of Neoral (mg/kg per day)	(ng/mL)		
Pediatric (≤15 y)	8.3 ± 3.9	150 ± 88	455 ± 374	1738 ± 1485	6.4 ± 3.4	124 ± 73	401 ± 157	1269 ± 415
Adult (>15 y)	2.6 ± 0.8	185 ± 108	652 ± 276	2200 ± 759	1.5 ± 0.5	118 ± 37	453 ± 197	1564 ± 613

C_0, trough blood concentration; C_2, blood concentration 2 hours after administration; AUC_{0-4}, mean area under the curve between trough and 4 hours after Neoral administration.

liver transplantation were biliary atresia (n = 6), liver cirrhosis due to hepatitis C virus (n = 3), fulminant hepatic failure (n = 3), familial amyloid polyneuropathy (n = 3), Alagille's syndrome (n = 2), and primary biliary cirrhosis (n = 1).

The initial tacrolimus dose of 0.05 mg/kg per day was adjusted to maintain whole blood trough concentrations (C_0) of 15 to 18 ng/mL during the first 2 weeks after transplantation, 10 to 15 ng/mL during the third and fourth weeks, and 5 to 10 ng/mL thereafter. All patients received a 20 mg/kg intravenous bolus of methylprednisolone before reperfusion of the liver graft. From postoperative day 1, methylprednisolone was tapered from 3 to 0.5 mg/kg within 7 days and thereafter to 0.06 mg/day at 6 months. The tacrolimus C_0 was measured by the microparticle enzyme immunoassay technique (Abbott Japan, Tokyo, Japan). When patients developed tacrolimus-related adverse effects, the tacrolimus was withdrawn and cyclosporine (twice a day orally) was commenced 12 hours later. The reasons for changing the medication were liver dysfunction (n = 6), renal dysfunction (n = 5), gastrointestinal toxicity (n = 3), diabetes (n = 2), neurotoxicity (n = 1), and hematological disorder (thrombotic thrombocytopenic purpura; n = 1), all of which were suspected to be tacrolimus-related. The mean tacrolimus C_0 at the onset of adverse reactions was 12.3 ± 5.0 (range 5.6 to 21.6) ng/mL.

The first Neoral dose ranged from 2 to 3 mg/kg per day in two divided doses. The doses of Neoral were titrated to maintain the cyclosporine C_0 within the target range as described elsewhere.[6] Following the change in their drug regimens, patients were evaluated every day for the first week; weekly for the next 3 weeks; and then at months 2, 3, 6, and 12 for therapeutic cyclosporine blood levels, acute rejection episodes, graft function, and assessment of the signs and symptoms that had led to tacrolimus withdrawal. Monitoring of the tacrolimus blood level was continued until it fell below 3 ng/mL. Each evaluation of cyclosporine blood levels included C_0, the 1-hour postdose blood concentration (C_1), C_2 and the 4-hour postdose blood concentration (C_4). The area under the time-blood cyclosporine concentration curve between C_0 and C_4 (AUC0 = 4) was measured by the linear trapezoidal rule. Correlations of single-point samples were done by Pearson's correlation test. In all subjects the efficacy of immunosuppression was assessed over the 6 months following the change to Neoral. The median follow-up time was 1001 days.

RESULTS

None of the 18 patients died during the follow-up period. Resolution or improvement of tacrolimus-related adverse effects was observed in all patients except three who had liver dysfunction. The time from withdrawal of the tacrolimus to resolution of the side effects was 1, 2, 3, 5, and 14 days for neurotoxicity; 8, 16, and 21 days for hepatotoxicity; 3, 5, and 9 days for gastrointestinal toxicity; 13 and 44 days for diabetes; 10 days for thrombotic thrombocytopenic purpura; and 3 days for nephrotoxicity. Two adult patients experienced rejection episodes—one at day 65 and another on day 201 after the change in drug regimen. All episodes responded to steroid pulse therapy.

Adequate trough concentrations were achieved with Neoral (Table 1). During the drug conversion phase, the blood concentrations of both tacrolimus and cyclosporine were monitored and the dose of Neoral titrated. For example, when the blood level of tacrolimus had declined to half the target level, the Neoral was titrated to achieve half the target cyclosporine trough level. As a result, no adverse effects from coadministration of the two drugs—including deterioration of renal dysfunction—were encountered. The maintenance doses differed considerably between pediatric and adult recipients (Table 1). Statistical analysis showed that C_2 correlated better with AUC0 = 4 (R = 0.970) than C_0 (R = 0.586), C_1 (R = 0.881), or C_4 (R = 0.768). However, in two infant recipients, the blood concentration curve did not peak at C_1 or C_2, but increased gradually to C_4. These two patients had undergone liver transplantation because of liver cirrhosis after a Kasai procedure for biliary atresia. The other three infant patients who underwent liver transplantation after a Kasai procedure had absorption profiles peaking at C_2. None of the recipients with biliary atresia developed gastrointestinal disorders or biliary complications.

DISCUSSION

We evaluated a protocol whereby the immunosuppressant drug therapy of 18 patients who had undergone liver transplantation was switched from tacrolimus to Neoral. The initial dose of Neoral was set relatively low at 2 to 3 mg/kg per day, and the drugs concentrations were monitored closely for 1 week. This resulted in fair outcomes without deterioration of nephropathy. We also investigated the pharmacokinetics of Neoral to assess the validity of C_2 monitoring with positive results. We were able to determine the target C_2 levels for liver transplant patients observing considerable differences in the requirements between children and adults (Table 1).

Neoral is a microemulsion formulation of cyclosporine with more consistent pharmacokinetic parameters and better bioavailability than the conventional oil-based formula-

tion of cyclosporine. Some studies have demonstrated that induction and maintenance of immunosuppression with Neoral are more effective than with Sandimmune, as demonstrated by a lower incidence of acute rejection with no additional toxicity. Our present study showed that C_2 may prove to be an excellent surrogate marker of AUC0 - 4, as described previously.[9] However, careful monitoring of C_2 is necessary for some infant recipients who have undergone the Kasai procedure for biliary atresia.

In summary, tacrolimus-related adverse effects can be treated successfully with a low incidence of rejection by switching patients to Neoral. We have determined the maintenance dose of Neoral in pediatric and adult liver transplant recipients as well as the recommended C_2 levels for drug monitoring.

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DELAYED DOMINO LIVER TRANSPLANTATION: USE OF THE REMNANT LIVER OF A RECIPIENT OF A TEMPORARY AUXILIARY ORTHOTOPIC LIVER TRANSPLANT AS A LIVER GRAFT FOR ANOTHER PATIENT

Domino liver transplantation (DLT) has been developed as a method for expanding the donor pool (*1*). In contrast, temporary auxiliary partial orthotopic liver transplantation (APOLT) has been used for selected liver diseases (*2*). In temporary APOLT, we routinely resect the left liver and transplant the donor's left liver, and thereafter ligate and divide the right portal vein. This causes hypertrophy of the graft in the recipient and atrophy of the remnant liver, which is then removed approximately 2 months after transplantation (*2*).

A 27-year-old man with familial amyloid polyneuropathy (patient 1) was referred to our institution for living-donor liver transplantation. Preoperative computed tomography of the donor candidate showed a left hepatic lobe volume of 322 mL, corresponding to only 28% of the recipient's standard liver volume. We decided to perform temporary APOLT for the patient, considering the safety of both donor and recipient. On July 4, 2001, the left hepatic lobe (segments I–IV according to Couinaud's nomenclature) was resected from patient 1 and the left liver graft (segments II–IV) was orthotopically transplanted. However, just after reperfusion, color-duplex ultrasonography demonstrated thrombosis of the graft hepatic vein and the portal vein. Continuous administration of heparin resulted in successful thrombectomy and reanastomosis, allowing recovery of graft perfusion. We then considered whether to ligate the right portal vein, as scheduled. Considering the possible recurrence of thrombosis and the need to modulate the portal venous flow to the recipient's remnant liver to induce compensatory hypertrophy of the left lobar graft, we decided not to ligate the trunk of the right portal vein, but to transect only the anterior branch of the right portal vein, leaving the posterior branch patent. The postoperative course was uneventful, and removal of the remnant native liver was planned 8 weeks after APOLT.

On August 10, 2001, a 61-year-old man with hepatocellular carcinoma (patient 2) was referred for possible living-donor liver transplantation. However, no suitable donor was available. We carefully discussed the adequacy of using the remnant liver of patient 1 as a graft for patient 2, on the basis of our experience with preoperative portal vein embolization for extended lobectomy of the liver (*3*). Taking care to avoid any coercion, we asked patient 1 whether he would be willing to approve use of the removed remnant liver as a graft for another patient. His reply was promptly affirmative. We then explained to patient 2 the delayed DLT procedure, including its untried aspects, uncertainty about the graft quality, and future onset of familial amyloid polyneuropathy. The patient expressed a strong desire to undergo this procedure. The entire process was reviewed and approved by the Ethical Committee of Shinshu University. On September 4, 2001, we removed the remnant right liver from patient 1 and transplanted it into patient 2. The postoperative courses in these patients were uneventful. The presented cases indicate that division of one of the two portal venous branches to the remnant right liver is feasible in APOLT and that delayed DLT could be an option in selected situations.

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Prognostic Significance of Mature Dendritic Cells and Factors Associated With Their Accumulation in Metastatic Liver Tumors From Colorectal Cancer

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Although dendritic cells (DCs) play an important role in tumor immunity, their prognostic significance and factors related to mature DCs have not been addressed in metastatic liver tumors. In surgically resected, paraffin-embedded tissue sections from 70 patients with colorectal liver metastasis, CD83 (a marker of mature DCs) positive cells and cancer cells positive for the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling assay were counted. Expression of gp96, which is considered to participate in the maturation of DCs, was also evaluated. CD83-positive cells were observed predominantly in the cancer invasive margin. Patients with CD83-positive cell counts of <2 per field had a significantly poorer prognosis (5-year survival rate 47.5% vs 23.1%; $P = 0.0184$). Patients with >0.83% apoptotic cancer cells had significantly higher numbers of CD83-positive cells (7.3 ± 7.3 vs 4.0 ± 5.1; $P = 0.039$). Patients with immunohistochemically positive gp96 expression in tumors had significantly higher numbers of CD83-positive cells than those with negative gp96 expression (6.0 ± 6.5 vs 1.4 ± 2.3; $P = 0.0108$). Patients with metachronous occurrence of liver metastasis had significantly higher numbers of CD83 positive cells than those with synchronous detection (6.3 ± 6.5 vs 3.9 ± 5.9; $P = 0.0313$). Although the number of apoptotic cancer cells, degree of tumor gp96 expression, and synchronous or metachronous occurrence of liver metastasis did not directly influence patient outcome, they did influence the number of CD83-positive cells in the cancer invasive margin, which was a significant prognostic factor in patients with colorectal liver metastasis. HUM PATHOL 35:1392-1396. © 2004 Elsevier Inc. All rights reserved.

Key words: **CD83, dendritic cell, apoptotic cancer cell, heat shock protein, gp96.**

Abbreviations: **DC, dendritic cell; HSP, heat-shock protein; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline containing 0.1% (v/v) Tween-20; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.**

Several studies have shown that high numbers of dendritic cells (DCs) in tumor tissue, including lung cancer, breast cancer, and head and neck cancer, correlate with good prognosis.[1-3] However, no study to date has investigated the prognostic significance of mature DCs in metastatic liver tumors.

The most potent antigen-presenting cells, DCs are central to the regulation, maturation, and maintenance of the cellular immune response against cancer. After taking up antigens, immature DCs differentiate into mature DCs that prime naive T cells and initiate antigen-specific T-cell responses to the cancer.[4] Killed tumor cells have been shown to be a source of tumor-associated antigens for processing and presentation by DCs. However, there remains some disagreement as to the optimal form of killed tumor cells for DC uptake, maturation, and capacity to stimulate antigen-reactive T cells via the separate major histocompatibility class I and II pathways.[5] Sauter et al[6] reported in an in vitro study that although immature DCs phagocytosed both apoptotic and necrotic tumor cells, only necrotic cells induced DC maturation. This study also found that the range of dying cell to DC ratio was limited, such that some mixture ratios failed to induce DC maturation and that large amounts of apoptotic cells induced DC death. Because human cancers involve both apoptotic and necrotic tumor cells, the ratio of apoptotic to necrotic tumor cells can vary widely, making the quantitative assessment of tumor necrosis difficult in human carcinoma tissue. There have not yet been any reports on the relationship between the degree of apoptotic tumor cells and mature DCs in in vivo cancer tissue.

Among several factors that induce DC maturation,[7-15] heat-shock proteins (HSPs) are the most likely candidates for this effect.[16] Surface expression of gp96 on tumor cells stimulates DC maturation in vitro and induces efficient T-cell priming and tumor rejection in vivo.[17] It also has been reported that HSPs are released from tumor lysate but not from apoptotic cells. On the other hand, Feng et al[18] reported that once stressed, HSPs were identified on apoptotic cells.

The aims of the present study were to evaluate the prognostic significance of CD83 (a marker of mature DCs) positive cell infiltration,[19-22] and clinicopathologic factors associated with their accumulation in patients with metastatic liver tumor.

PATIENTS AND METHODS

Between January 1990 and January 2004, 118 patients underwent macroscopically curative hepatic resection due to colorectal liver metastasis at the First Department of Surgery, Shinshu University Hospital. Informed consent was obtained preoperatively from each patient to use part of the resected

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cancer lesion for research. Of the 113 patients, more than 5 years had passed after initial hepatectomy in 70. The patient group comprised 44 men and 26 women with a mean age of 61.1 years (range, 33 to 82 years). None of the patients received adjuvant chemotherapy. After discharge, all patients were followed up at our outpatient clinic on a monthly or bimonthly basis.

Clinicopathologic data on these patients were also reviewed retrospectively. As prognostic factors, age, sex, presence or absence of other distant metastasis (lung) at initial hepatectomy, number and maximum diameter of metastatic liver tumors, histological differentiation, presence or absence of cancerous exposure at the transected margin, and synchronous or metachronous detection and resection of liver metastasis were examined.

Immunohistochemistry for CD83 and gp96

Formalin-fixed, paraffin-embedded tissues were cut into 4-μm-thick sections and mounted on glass slides coated with poly-L-lysine. Deparaffinized rehydrated sections were incubated in 0.01 N HCl and 0.5% (v/v) pepsin for 20 minutes at 37°C to allow antigen retreval of CD83. Endogenous peroxidase activity was blocked by incubation for 10 minutes with methanol containing 3% (v/v) hydrogen peroxide. Washing, blocking, and immunoreactivity signal amplification were performed using a tyramide signal amplification kit (NEN Life Science Products, Boston, MA) according to the manufacturer's instructions. Subsequently, primary antibodies were applied and left overnight at 4°C for CD83 and 1 hour at room temperature for gp96. The following primary antibodies were used: anti-CD83 (HB15A17.11, 1:1000 dilution; Serotec, Oxford, UK) and anti-gp96 (1:2000 dilution; NeoMarkers, Fremont, CA). Antibody binding was visualized using 3,3'-diaminobenzidine. After rinsing in water, cell nuclei were counterstained with hematoxylin, and the sections were dehydrated and coverslipped. As a negative control, normal mouse IgG (mouse primary antibody control; Zymed, San Francisco, CA) was used as the primary antibody.

CD83-positive cells were distributed predominantly in the invasive margin. Screening was performed at low magnification to first identify the 10 areas with the greatest numbers of CD83-positive cells in the invasive margin, then count the number of CD83-positive cells in each of these areas at high magnification (× 400).

Immunohistochemical staining for gp96 was evaluated by 2 independent observers who were blinded to the source of the specimens, and the entire area of each section was observed. Immunoreactivity of cancer or noncancer cells for gp96 was classified as negative if ≤10% of the total number of cancer or noncancer cells were positive, and positive if >10% of the total number of cancer or noncancer cells were positive.

Western Blot Analysis of gp96

Immediately after hepatectomy, samples obtained from both cancerous and noncancerous areas were snap-frozen in an acetone bath cooled in liquid nitrogen, and the specimens were then stored at −80°C. Liver tissue was homogenized in a buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol NaCl, 1% (v/v) Nonidet P-40, 0.1% (v/v) sodium dodecyl sulfate, 1% (w/v) sodium deoxycholate, 2 mmol EDTA, 1 mmol phenylmethyl sulfonyl fluoride, 2 mg/L aprotinin, 10 mg/L leupeptin, and 5 g/L pepstatin. The homogenates were centrifuged at 12,000 × g for 10 minutes at 4°C, and the supernatants were collected. The protein concentration was measured by bicinchoninic acid protein assay (Pierce, Rockford, IL). The same amounts of protein from liver homogenates were dissolved in sample buffer consisting of 25 mmol Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (v/v) sodium dodecyl sulfate, 0.02% (w/v) bromophenol blue, and 3% (v/v) 2-mercaptoethanol, loaded on 8% (w/v) polyacrylamide gels, and electrophoresed. The proteins were transferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA) by electroblotting. The membranes were blocked for 1 hour at room temperature with 5% (w/v) nonfat dried milk and 0.1% (w/v) bovine serum albumin in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 (TBS-T), then incubated for 1 hour with an anti-grp94 antibody diluted in TBS-T containing 5% (v/v) fetal bovine serum. After 3 washings in TBS-T, the membranes were incubated for 1 hour with peroxidase-conjugated rabbit anti-rat antibody (Dako, Glostrup, Denmark) diluted in TBS-T containing 5% (v/v) fetal bovine serum. After washing in TBS-T, blots were developed by enhanced chemiluminescence (Amersham) and exposed to X-ray film (RX-U; Fuji, Kawasaki, Japan).

Apoptotic Cancer Cell Count

To estimate apoptotic cancer cells, deparaffinized rehydrated sections were stained by the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay[25] with some modifications. Briefly, formalin-fixed, paraffin-embedded sections were dewaxed, rehydrated, and digested with 20 μg/mL proteinase K (Sigma) for 15 minutes. Endogenous peroxidase was blocked by treatment with 0.3% (v/v) hydrogen peroxide. Sections were then rinsed in water and incubated with 50 μL terminal deoxynucleotidyl transferase buffer (30 mmol/L Tris-HCl [pH 7.2], 140 mmol/L sodium cacodylate, 1 mmol/L cobalt chloride) containing 8.3 U terminal deoxynucleotidyl transferase (Boehringer Mannheim, Mannheim, Germany) and 0.83 nmol biotinylated 16-deoxyuridine triphosphate (Boehringer Mannheim) in a moist chamber at 37°C for 60 minutes. Sections were then rinsed and incubated with horseradish peroxidase–conjugated streptavidin (Dako) diluted 1:500 in 0.01 mol/L Tris-HCl (pH 7.5) and 150 mmol/L NaCl (TBS) containing 1% (v/v) bovine serum albumin (Sigma, St. Louis, MO) for 30 minutes at room temperature, then rinsed in TBS and stained with diaminobenzidine. The percentage of apoptotic cancer cells was calculated by TUNEL positivity in 5 randomly chosen high-power (× 400) fields on each section.

Statistical Analysis

Data are shown as mean ± standard deviation. The Mann-Whitney *U*-test was used to compare the 2 groups. Survival curves were calculated by the Kaplan-Meier method, and statistical significance was tested by the log-rank test. Multiple regression analysis was performed using the Cox proportional hazards model. Variables to be entered into the multivariate analysis were chosen on the basis of the results of univariate analysis. A stepwise procedure and a likelihood ratio test were used to select the variables for the final model. All analyses were performed using the Statview 5.0 statistical software package (Abacus Concepts, Berkley, CA).

RESULTS

CD83-positive cells were observed predominantly in sinusoid-like vessels very close to cancer cells and among infiltrating cells in the cancer invasive margin (Fig 1). When patients were divided into 2 groups based on the median value of CD83-positive cell count

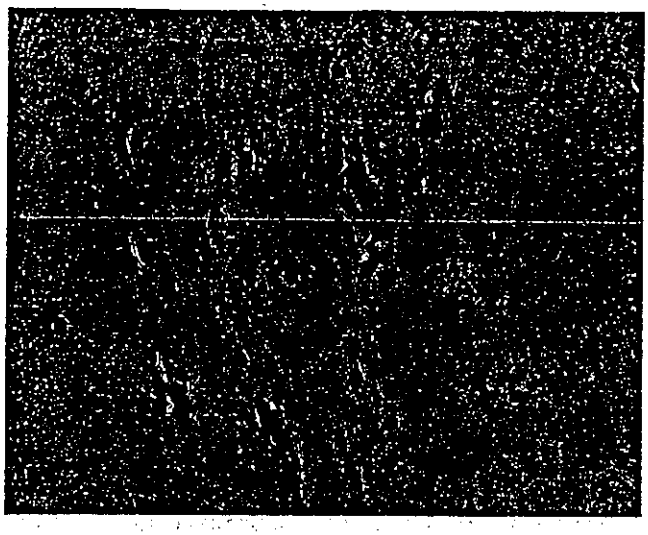

FIGURE 1. CD83-positive cells distributed predominantly in an invasive margin. (Original magnification × 400.)

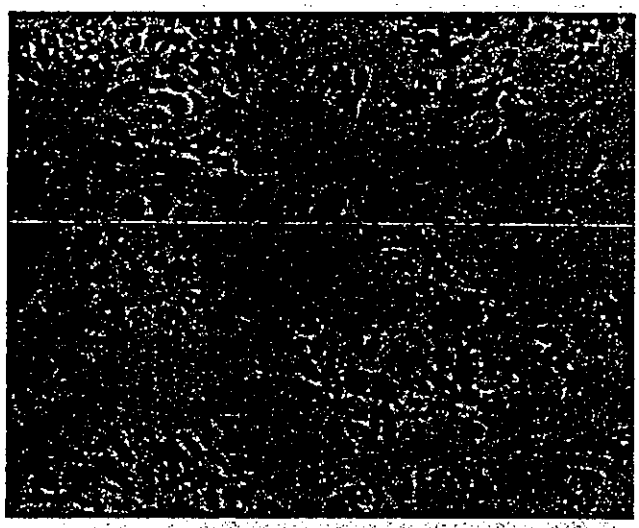

FIGURE 3. TUNEL-positive apoptotic tumor cells in colorectal liver metastasis. (Original magnification × 400.)

over a total of 700 areas (ie, 10 areas from 70 patients), patients with CD83-positive cell counts of <2 per field (n = 26) had a significantly poorer prognosis than patients with ≥2 CD83-positive cells per field (n = 44) (5-year survival rate, 47.5% vs 23.1%; $P = 0.0184$) (Fig 2).

The mean percentage of apoptotic cancer cells per slide was 0.83%, with a range of 0 to 3.9% (Fig 3). Patients with >0.83% apoptotic cancer cells (n = 32) had a significantly higher number of CD83-positive cells than patients with ≤0.84% apoptotic cancer cells (n = 38) (7.3 ± 7.3 vs 4.0 ± 5.1; $P = 0.039$). However, the percentage of apoptotic cancer cells itself had no influence on patient survival.

Western blot analysis demonstrated gp96 expression in both cancer and noncancer regions (Fig 4); gp96 immunostaining also demonstrated positivity in both cancer and noncancer regions. Immunohistochemically, 61 patients exhibited positive gp96 expression in tumor tissue (Fig 5), and these patients had a significantly higher number of CD83-positive cells than the remaining 9 patients who exhibited negative gp96 expression (6.1 ± 6.6 vs 1.4 ± 2.3; $P = 0.0107$). Noncancerous gp96 expression had no influence on the number of CD83-positive cells. Also, gp96 itself was not a significant prognostic factor.

Univariate analysis demonstrated that patients with mucinous tumor ($P < 0.0001$), a positive surgical margin ($P = 0.0121$), and lung metastasis ($P = 0.001$) had significantly poorer prognosis. However, age, sex, number of liver tumors, maximum tumor diameter, and synchronous or metachronous detection did not significantly correlate with patient survival after initial hepatectomy (Table 1). Patients with metachronous resection had a significantly higher number of CD83-positive cells than those with synchronous resection (6.3 ± 6.5 vs 3.9 ± 5.9; $P = 0.0313$), although the remaining factors were not related to CD83-positive cell count. Cox multivariate regression analysis demonstrated that lung metastasis, histological differentiation, and number

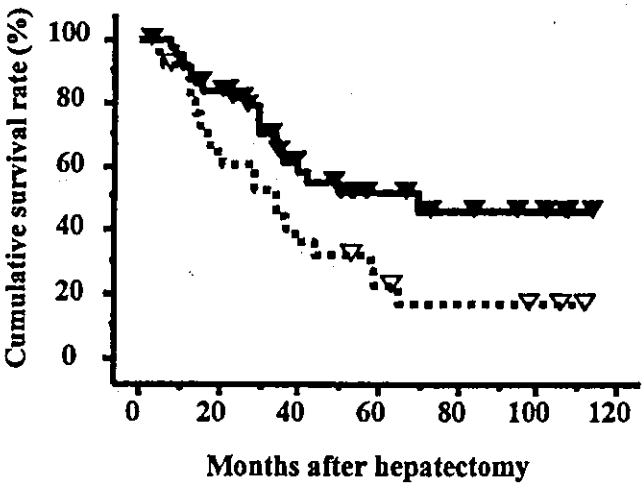

FIGURE 2. Comparison of the cumulative survival rate between patients with CD83-positive cell counts of <2 per field (dotted line, n = 26) and patients with ≥2 CD83-postive cells per field (solid line, n = 44).

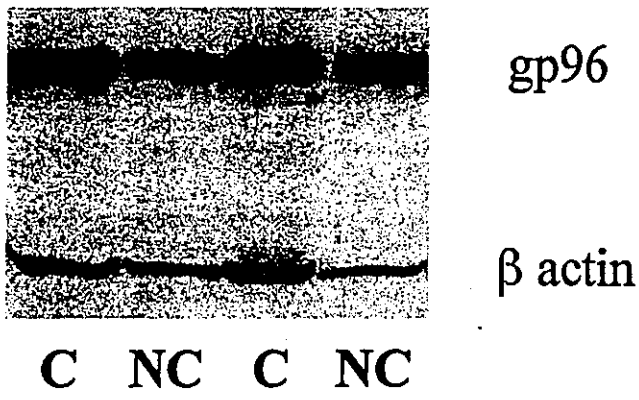

FIGURE 4. Western blot of gp96 and β-actin in cancer (C) and noncancer tissues (NC).

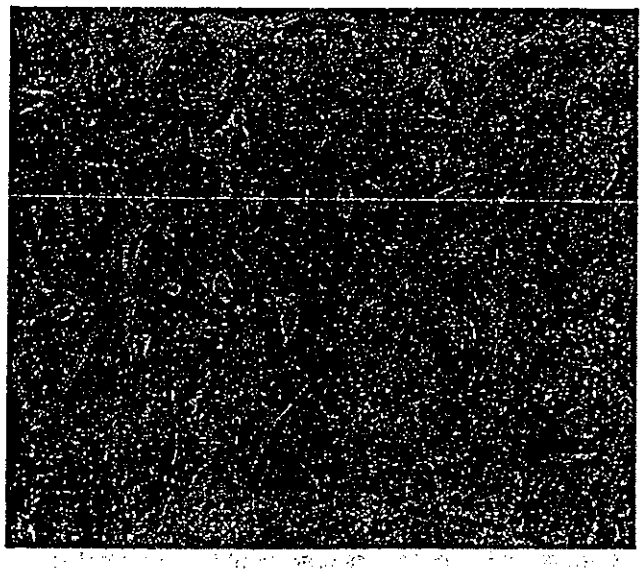

FIGURE 5. Immunostaining of gp96-positive cancer tissue. (Original magnification × 100.)

of CD83-positive cells in the cancer invasive margin were significant independent prognostic factors (Table 2).

DISCUSSION

An abundance of intratumor DCs is associated with better patient outcomes for a variety of carcinomas, including cervical, colorectal, gastric, lung, and nasopharyngeal cancers.[1-3] CD83 is widely used in in vitro studies as a marker for mature DCs.[19-22] Bell et al[24] reported that immature DCs reside within the tumor, whereas mature DCs tend to be located in the peritumoral areas in breast carcinoma. Suzuki et al[25] reported that in the invasive margins of colorectal cancer stroma, mature CD83-positive DCs form clusters with T cells to promote T-cell activation and the development of tumor-specific immunity. However, no study has investigated the prognostic significance of mature DCs in metastatic liver tumors. The present study demonstrated that patients with mature DC counts of <2 per field had a significantly poorer prognosis. Sauter et al[6] reported that although immature DCs phagocytosed both apoptotic and necrotic cells, only necrotic cells induced DC maturation. Their study also described that the range of dying cells to DC ratio was limited, such that some mixture ratios failed to induce DC maturation and that large amounts of apoptotic cells induced DC death.[6] The uptake of apoptotic cells may be involved in the production of anti-inflammatory signals that establish peripheral self-tolerance.[26-28] Pietra et al[29] reported that tumor cells in early phases of apoptosis inhibited DC maturation, whereas cells in late apoptosis or even primary necrosis delivered a partial maturation signal. In vitro studies have yielded controversial results. Also, human cancers involve both apoptotic and necrotic tumor cells, and the ratio of apoptotic to necrotic tumor cells can vary widely. To date, there have been no reports on the relationship between apoptotic tumor cells and mature DCs in in vivo cancer tissue. Our study found that the mean percentage of apoptotic cancer cells was only 0.84%. This did appear to influence the number of CD83-positive cells, although it was not a prognostic factor. This result suggests that mature DCs are positively associated with apoptotic cancer cells in in vivo human cancer tissue.

Several factors induce DC maturation. These include microorganisms,[7-9] presence of the CD40 ligand on activated T cells,[10-12] cytokines (eg, TNF-α, IL-1β), bacterial and viral products,[13-15] and nucleotides, but HSPs are the most likely candidates for this effect.[16] Surface expression of gp96 on tumor cells stimulates DC maturation in vitro and induces efficient T-cell priming and tumor rejection in vivo.[17] In the present study, gp96 itself was not found to be a prognostic factor. However, in cancers immunohistochemically positive for gp96, a significantly higher number of

TABLE 1. Univariate Analysis of Predictive Factors

Factor	Number of patients	5-year survival rate (%)	*P* value
Age (years)			0.6625
<62	35	42.7	
≥62	35	33.6	
Sex			0.3594
Male	44	38.3	
Female	26	38.5	
Histological types			<0.0001
Well differentiated	30	49.6	
Moderately differentiated	35	34.3	
Mucinous	5	0	
Number of metastatic liver tumors			0.3266
1	31	41.2	
≥2	39	35.7	
Maximum tumor diameter (mm)			0.3254
<47	42	32.5	
≥47	28	46.4	
Lung metastasis			0.001
Presence	16	12.5	
Absence	54	45.7	
Occurrence of metastasis			0.4822
Synchronous	24	29.2	
Metachronous	46	42.9	
Surgical margin			0.0121
Positive	20	15.0	
Negative	50	47.3	

TABLE 2. Cox's Multivariate Regression Analysis

Variables	χ^2	*P* value	Hazard ratio	95% confidence interval
Histological type	20.362	<0.0001		
Moderately differentiated	8.345	0.0039	2.53	1.348–4.751
Mucinous	18.5	<0.0001	12.223	3.906–38.252
CD83-positive cell count < 2	8.227	0.0041	2.385	1.317–4.319
Absence of lung metastasis	9.773	0.0018	0.33	0.171–0.638

CD83-positive DCs was observed at the invasive margin than in gp96-negative cancers. This finding suggests that cancer gp96 expression is related to CD83-positive DCs in patients with metastatic liver tumors.

The effect of the time interval between the primary procedure and liver resection on the patient's prognosis remains controversial. The plausible causes of better prognosis in patients with metachronous occurrence of liver metastasis have not been defined, even though the prognostic significance of metachronous occurrence of liver metastasis has been described.[30-33] The present study has revealed that although synchronous or metachronous resection of liver metastasis did not influence prognosis after initial hepatectomy, patients who underwent metachronous resection and had a disease-free interval from primary to metastasis had a significantly higher number of mature DCs. This finding suggests a difference in host immunity between patients with synchronous and metachronous occurrence of liver metastasis. This difference in mature DC count might be related to the prognostic difference between these patient groups, as demonstrated by some previous studies.[30-33]

Although the number of apoptotic cancer cells, tumor gp96 expression, and synchronous or metachronous occurrence of liver metastasis had not influence on patient's outcome, they did appear to influence the number of CD83-positive cells in the cancer invasive margin. This was a significant prognostic factor in patients with colorectal liver metastasis.

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Dendritic Cells, T-Cell Infiltration, and Grp94 Expression in Cholangiocellular Carcinoma

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Although dendritic cells (DCs) play an important role in tumor immunity, there have been no reports on their role in cholangiocellular carcinoma (CCC). In 26 formalin-fixed, paraffin-embedded tissue sections from patients with CCC, cells positive for CD83 (a marker of mature DCs), CD1a (a marker of immature DCs), and CD8 and CD4 (T cell markers) were counted, and expression of glucose-regulated protein (grp) 94, which is considered to participate in the maturation of DCs, was evaluated by immunohistochemistry and Western blot analysis to study the relationship between their expression and patients' disease outcome. The number of CD83-positive DCs at the invasive margin of CCCs correlated significantly with the number of CD8-positive or CD4-positive T cells in the cancerous region and was significantly higher in grp94-positive cancer than in grp94-negative cancer ($P = 0.0006$). CD83-positive patients (positive cells in invasive margin > 12.4/field) had both a significantly lower incidence of lymph node metastasis (23.1% vs 69.2%; $P = 0.0206$) and a better outcome than CD83-negative patients ($P < 0.001$). We conclude that mature DCs are distributed predominantly at the invasive margin of cancers, and a significantly higher number of mature DCs at the invasive margin are observed in patients with grp94-positive cancer cells. Mature DCs may enhance CD8- and CD4-positive cell infiltration into cancers and improve prognosis in patients with CCC, due in part to abatement of lymph node metastasis. HUM PATHOL 35:881-886. **© 2004 Elsevier Inc. All rights reserved.**

Key words: **cholangiocellular carcinoma, dendritic cells, CD83, grp94.**

Abbreviations: **CCC, cholangiocellular carcinoma; CR, cancerous region; DC, dendritic cell; grp94, glucose-regulated protein 94; HSP, heat-shock protein; IM, invasive margin; NC, noncancerous region; TBS-T, Tris-buffered saline containing 0.1% Tween-20; TIL, tumor-infiltrating lymphocyte.**

Dendritic cells (DCs) are the most potent antigen-presenting cells and are central to the regulation, maturation, and maintenance of a cellular immune response to cancer. After taking up antigens, immature DCs differentiate into mature DCs that prime naive T cells and initiate antigen-specific T-cell responses to the cancer.[1] The T-cell antigen receptors recognize fragments of antigens bound to molecules of major histocompatibility complex classes I and II on the surface of DCs, which stimulate CD8-positive cytotoxic T lymphocytes and CD4-positive helper T cells.[2]

Several studies have shown that the presence of a high number of DCs in tumor tissue (eg, lung cancer, breast cancer, head and neck cancer) correlates with a good prognosis.[3-5] Among several DC-specific and maturation-associated markers, CD83 is a marker for mature DCs[6] and CD1a is a marker for immature DCs.[7,8]

The ability to present exogenous antigens through a process called "cross-presentation" is a key feature of DCs. Peptides chaperoned by heat-shock protein (HSP), like glucose-regulated protein 94 (grp94), which is resident in the endoplasmic reticulum, can be presented to cytotoxic T lymphocytes by DCs. Such presentation requires the uptake of grp94 via a cell surface receptor, CD91, which is expressed by DCs.[9-11]

Until now, the emergence and prognostic significance of DCs and grp94, which stimulate T lymphocytes and may play a central role in the antitumor immune response, have not yet been examined in cholangiocellular carcinoma (CCC). The aims of this study were, therefore, to evaluate the infiltration of mature DCs, CD8-positive T cells, CD4-positive T cells, and tumor grp94 expression in patients with CCC.

MATERIALS AND METHODS

Patients

Between 1992 and 2002, 26 patients underwent hepatectomy for CCC at the First Department of Surgery, Shinshu University Hospital, leaving no macroscopic evidence of residual cancer. This group comprised 15 men and 11 women with a mean age of 67 years (range, 52 to 81 years). After discharge, all of the patients were followed at our outpatient clinic on a monthly or bimonthly basis. The median follow-up time was 17 months (range, 2 to 47 months). Permission was obtained preoperatively from each patient to use part of the resected tumor lesion for research.

Immunohistochemistry of CD83, CD1a, grp94, CD4, and CD8

Formalin-fixed, paraffin-embedded tissues were cut into 4-μm-thick sections and mounted on glass slides coated with poly-L-lysine. The endogenous peroxidase activity was blocked for 30 minutes with 3% (v/v) hydrogen peroxidase. We used the tyramide signal amplification system (NEN Life Science

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Products, Boston, MA) according to the manufacturer's instructions. The sections were preincubated with TNB blocking buffer containing 0.1 mol Tris-HCl (pH 7.5), 0.15 mol NaCl, and 0.5% (w/v) blocking reagent (supplied in a kit). Subsequently, primary antibodies were applied and left overnight at 4°C. The primary antibodies used were anti-CD83 (Serotec, Oxford, UK), anti-CD1a (Serotec), anti-grp94 (Neo-Markers, Fremont, CA), anti-CD4 (Novocastra, Newcastle Upon Tyne, UK), and anti-CD8 (Dako, Glostrup, Denmark). The sections were washed in TNT wash buffer containing 0.1 mol Tris-HCl (pH 7.5), 0.15 mol NaCl, and 0.05% (v/v) Tween 20. Antibody binding was visualized using 3,3'-diaminobenzidine. After rinsing in water, cell nuclei were counterstained with hematoxylin, and the sections were dehydrated and coverslipped.

For double immunostaining, sections were first incubated in 0.01 mol (pH 6.0) or 1 mmol (pH 8.0) ciytric acid buffer for 10 minutes after staining with alkaline phosphatase–conjugated streptavidin (Dako). The second step for the immunohistochemistry was done as described earlier using diaminobenzidene as the stain. As a negative control, normal mouse IgG (mouse primary antibody control; Zymed, San Francisco, CA) was used as the primary antibody.

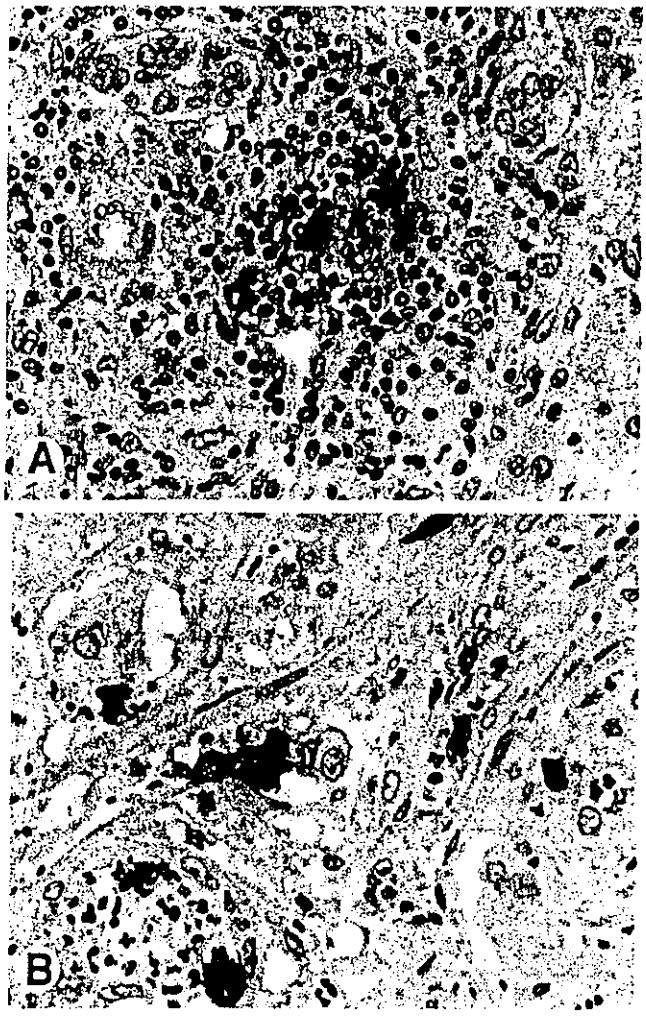

FIGURE 1. Immunostaining for CD83 in cancer tissues of CCC. (A) CD83-positive DCs at the IM. (B) CD1a-positive DCs in the CR. (Original magnification × 400.)

In 5 randomly chosen high-power fields (× 400) from 3 areas (CR, cancerous region; IM, invasive margin; and NC, noncancerous region), the numbers of cells positive for CD83, CD1a, CD8, and CD4 were counted using an Olympus BX50 microscope (Olympus, Lake Success, NY) and the same microscope objective. The immunohistochemical staining for grp94 was evaluated by 2 independent observers who were blinded to the source of the specimens, and the entire area of each section was observed. Immunoreactivity of cancer and noncancer cells for grp94 was classified as negative (−) if ≤5% of the total number of cancer or noncancer cells were positive, and as positive (+) if >5% of the total number of cancer or noncancer cells were positive.

Western Blot Analysis of grp94

Immediately after hepatectomy, samples obtained from both cancerous and noncancerous areas were snap-frozen in an acetone bath cooled in liquid nitrogen, and the specimens were then stored at −80°C. Liver tissue was homogenized in a buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol NaCl, 1% (v/v) Nonidet P-40, 0.1% (v/v) sodium dodecyl sulfate, 1% (w/v) sodium deoxycholate, 2 mmol EDTA, 1 mmol phenylmethyl sulfonyl fluoride, 2 mg/L aprotinin, 10 mg/L leupeptin, and 5 g/L pepstatin. The homogenates were centrifuged at 12,000 × *g* for 10 minutes at 4°C, and the supernatants were collected. The protein concentration was measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). The same amounts of protein from liver homogenates were dissolved in sample buffer (25 mmol Tris-HCl [pH 6.8], 10% [v/v] glycerol, 2% [v/v] sodium dodecyl sulfate, 0.02% [w/v] bromophenol blue, and 3% [v/v] 2-mercaptoethanol), loaded on 8% (w/v) polyacrylamide gels, and electrophoresed. The proteins were transferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA) by electroblotting. The membranes were blocked for 1 hour at room temperature with 5% (w/v) nonfat dried milk and 0.1% (w/v) bovine serum albumin in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBS-T), and then incubated for 1 hour with an anti-grp94 antibody diluted in TBS-T containing 5% (v/v) fetal bovine serum. After 3 washings in TBS-T, the membranes were incubated for 1 hour with peroxidase-conjugated rabbit anti-rat antibody (Dako) diluted in TBS-T containing 5% (v/v) fetal bovine serum. After washing in TBS-T, blots were developed by enhanced chemiluminescence (Amersham) and exposed to X-ray film (RX-U; Fuji, Kawasaki, Japan).

Statistical Analysis

The Mann-Whitney U-test and Spearman's rank correlation were used for statistical analyses. The χ^2 method with Yates's correction or Fisher's exact test was used for qualitative variables. The survival curves were calculated by the Kaplan-Meier method and compared using the log-rank test. Differences with a P value <0.05 were considered statistically significant.

RESULTS

CD83-positive cells (Fig 1A) were distributed predominantly in the IM (Fig 2), but CD1a-positive cells (Fig 1B) were observed only in the CR. Double immunostaining of CD83 and CD8 or of CD83 and CD4 (Fig 3A and B) showed each CD83-positive cell surrounded

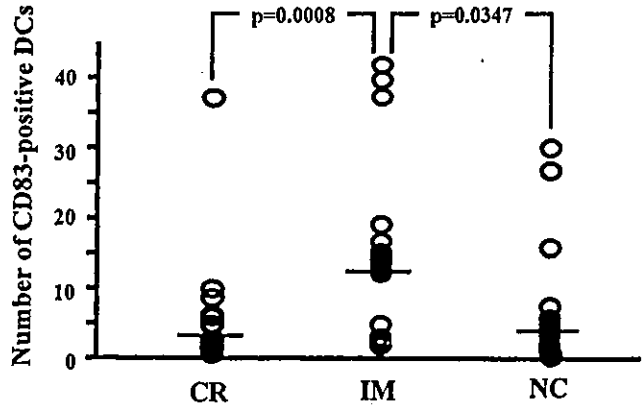

FIGURE 2. Predominant distribution of CD83-positive cells in the IM.

by CD8- or CD4-positive cells. The number of CD83-positive cells in the IM correlated significantly with the number of CD8-positive (Spearman's rank correlation coefficient = 0.507; $P = 0.0113$) or CD4-positive cells in the CR (Spearman's rank correlation coefficient = 0.598; $P = 0.0028$). CD8-positive patients (positive cells in CR >16/field) or CD4-positive patients (positive cells in CR >26/field) had significantly better prognoses than CD8-negative ($P = 0.0407$) or CD4-negative patients ($P = 0.0024$).

Western blot analysis (Fig 4) and immunostaining for grp94 (Fig 5) revealed grp94 expression in both CR and NC. However, the number of CD83-positive DCs in IM did not differ between patients with positive grp94 expression in NC and those with negative grp94 expression in NC ($P = 0.109$). Patients with grp94-positive cancer cells had a significantly high number of CD83-positive DCs in IM than those with grp94-negative cancer cells ($P = 0.0006$) (Fig 6), although grp94 itself was not a significant prognostic factor.

When patients were divided by the TNM classification,[12] the stage II patients had significantly longer survival than the stage IIIC patients ($P < 0.01$) (Fig 7).

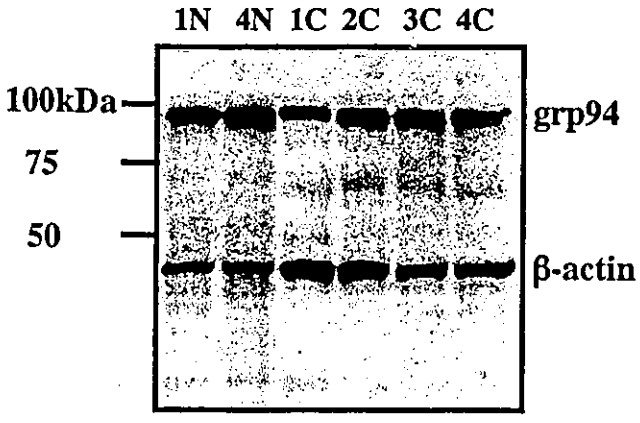

FIGURE 4. Western blot of grp94 and β-actin in cancerous (lanes 1C to 4C) and noncancerous tissues (lanes 1N and 4N). All samples show a strong band at 94 kDa, indicating grp94.

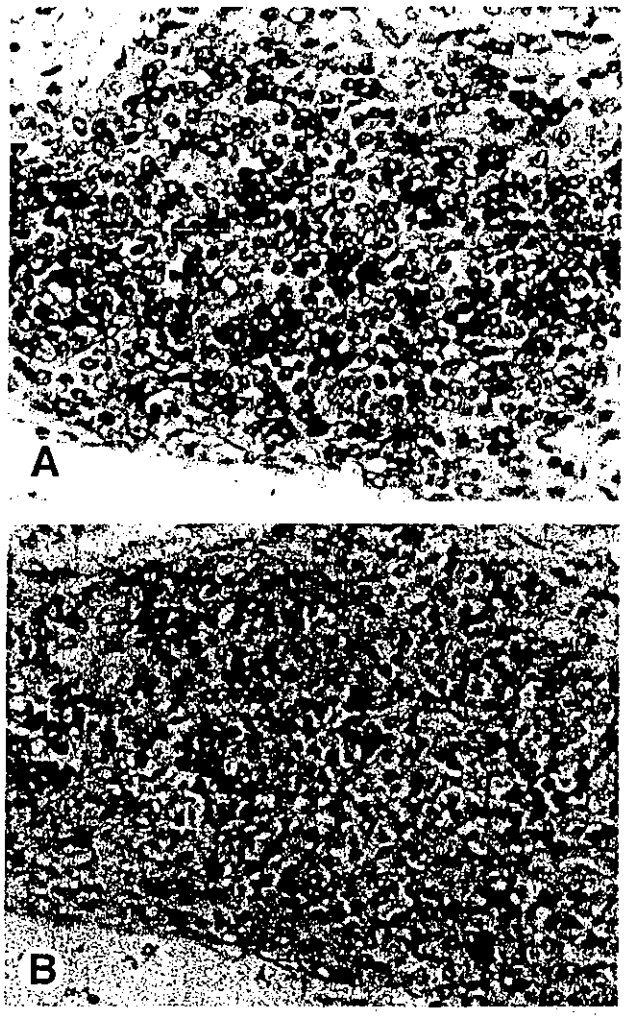

FIGURE 3. Double immunostaining of CD83 and CD8, and CD83 and CD4. (A) Double immunostaining of CD83 (pink) and CD8 (brown). (B) Double immunostaining of CD83 (pink) and CD4 (brown). (Original magnification × 400.)

Also, the stage II patients had significantly more CD83-positive DCs at the invasive margin than did the stage IIIC patients ($P = 0.0296$) (Fig 8).

When patients were divided into 2 groups (positive or negative) based on the median value of each factor, CD83-positive patients (positive cells in IM >12.4/field), whose CD83-positive cell count was 20.3 ± 11.1 (mean ± standard deviation), had both a significantly lower incidence of lymph node metastases (23.1% vs 69.2%; $P = 0.0206$) and better prognosis than CD83-negative patients (CD83-positive cell count, 2.7 ± 3.3) ($P < 0.001$) (Fig 9). CD1a-positive patients (positive cells in CR > 5.3/field) had also significantly better prognoses than CD1a-negative patients ($P = 0.0119$). The number of CD1a-positive cells in CR was significantly correlated with that of CD83-positive cells in IM (Spearman's rank correlation coefficient = 0.671; $P < 0.001$). Other clinicopathologic factors, including age, sex, preoperative serum carcinoembryonic antigen and carbohydrate antigenic determinant 19-9 levels,

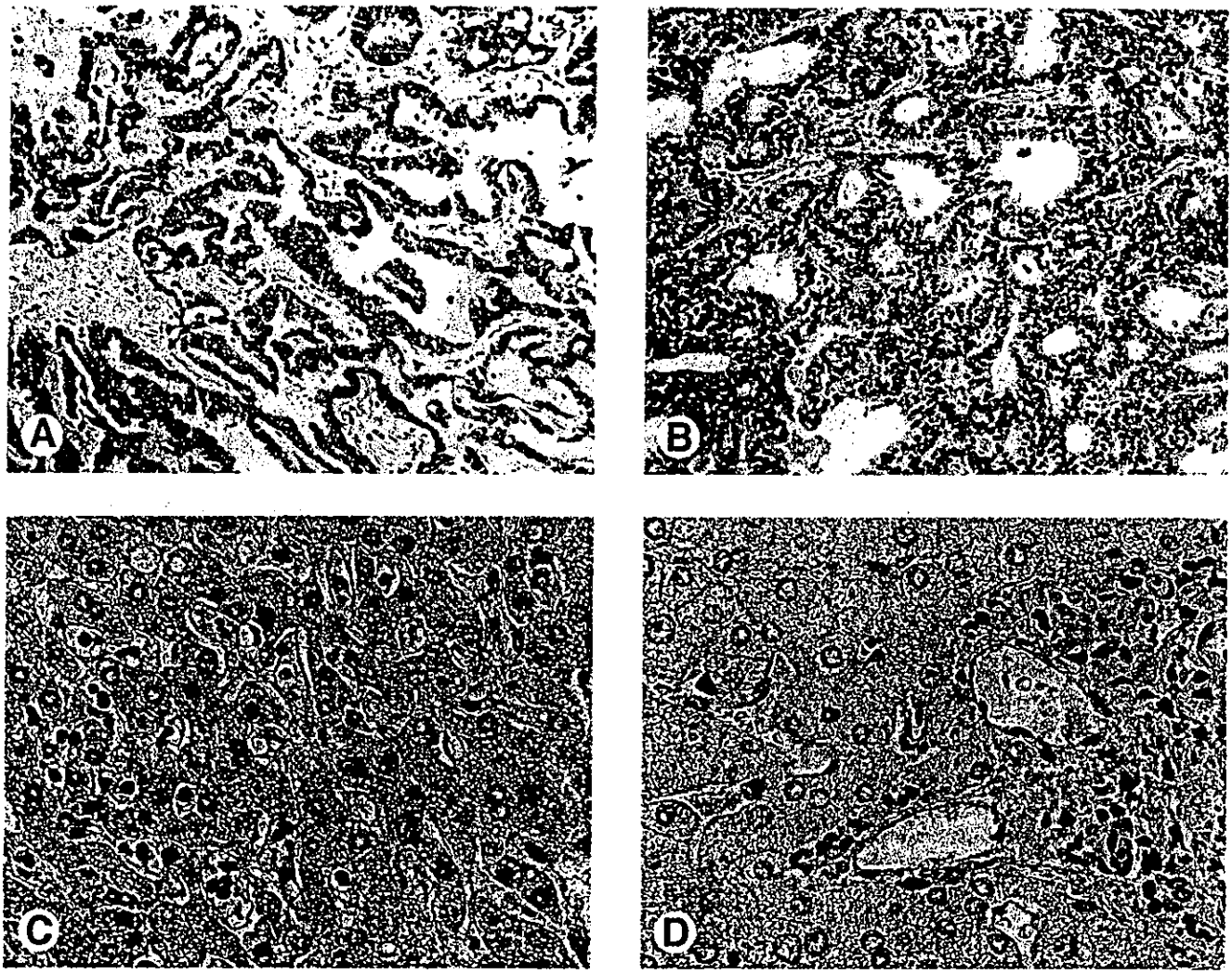

FIGURE 5. Immunostaining of grp94 of the tumor. (A) Immunostaining of grp94-positive CR. (B) Immunostaining of grp94-negative CR. (C) Immunostaining of grp94-positive NC. (D) Immunostaining of grp94-negative NC. (Original magnification × 400.)

tumor diameter, histological differentiation, vascular invasion, microscopic intrahepatic metastasis, and residual cancer at the transection margin showed no significant difference between CD83 or CD1a-positive and -negative patients.

DISCUSSION

CCC is the second most common primary malignancy of the liver. Its resectability rate is extremely low, but surgical resection is the only definitive treatment,[13,14] because the nonsurgical methods available to date have failed to change the outcomes. Therefore, there is a need to investigate new treatment modalities for CCC. An abundance of intratumor DCs is associated with a better outcome in patients with various of carcinomas, including cervical, colorectal, gastric, lung and nasopharyngeal cancers.[2-4] However, there has been no report on the relationship between the number of CD83-positive DCs or CD1a-positive DCs and survival in patients with CCC.

CD83 has been used widely for in vitro studies as a marker of mature DCs.[15-18] Bell et al[19] reported that in breast carcinoma immature DCs reside within the tumor, whereas mature DCs are located in peritumoral areas. Suzuki et al[20] reported that in the invasive margin of colorectal cancer stroma, mature CD83-positive DCs form clusters with T cells to promote T-cell activation for the generation of tumor-specific immunity. The present study also showed that in patients with CCC, the distribution of CD83-positive DCs differs from that of CD1a-positive DCs. CD83-positive DCs were located predominantly in the invasive margin, whereas CD1a-positive DCs were located only in the cancerous region. Immature DCs take up tumor-specific antigen, migrate to lymphoid tissue, and undergo maturation there. However, so far, no report has described the prognostic significance of immature DCs in the cancer-

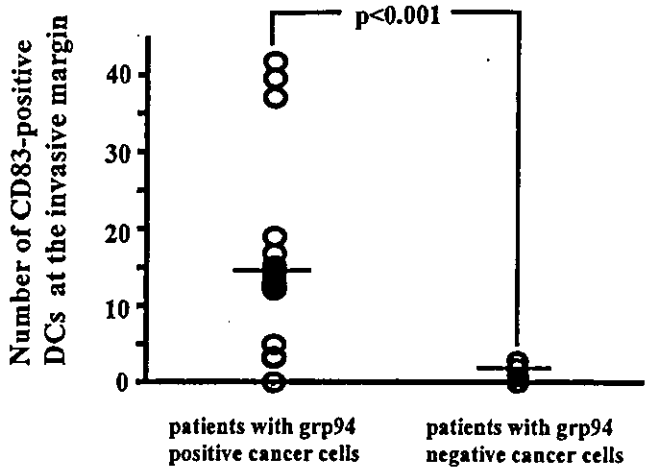

FIGURE 6. Numbers of CD83-positive DCs at the IM in patients with grp94-positive and grp-negative cancer cells. (14.7 ± 12.0 vs. 0.8 ± 1.3 cells/high power field; $P < 0.001$.)

ous region and mature DCs in the peritumoral area. The present study showed that the number of immature DCs in the cancerous region is correlated with that of mature DCs at the invasive margin and that both are significant prognostic factors, suggesting a potential relationship between DCs in the cancerous region and those at the invasive margin. However, this morphological analysis alone did not directly prove that immature DCs mature in the cancerous region and migrate to the invasive margin. The present study also showed that more advanced staged patients had fewer CD83-positive cell count, suggesting that a host immune response depends negatively on cancer progression.

Although previous studies showed that a greater abundance of tumor-infiltrating lymphocytes (TILs) and a stronger proliferative ability of TILs are associated with a better survival of patients with cancer,[21-22] including colorectal and renal cell carcinoma, there have been no reports on the relationship between TILs and DCs in patients with CCC. The present results obtained by double-immunostaining for CD83 and CD8 or CD83 and CD4 revealed that CD83-positive cells

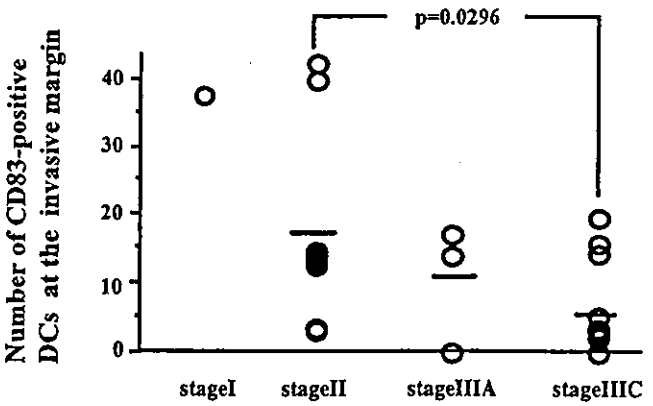

FIGURE 8. Numbers of CD83-positive DCs at the IM in stage I, II, IIIA and IIIC patients divided by TNM classification.

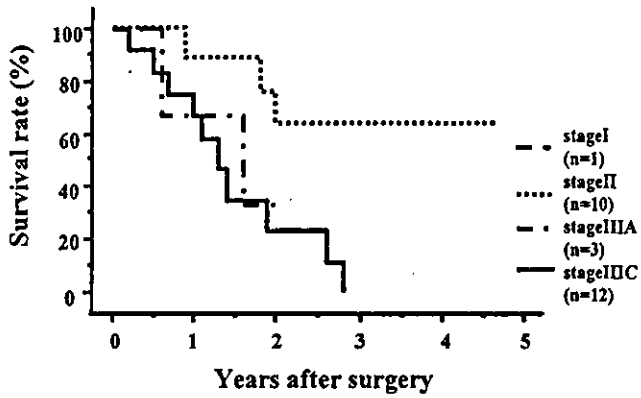

FIGURE 7. Five-year survival curves in patients with stages I, II, IIIA, and IIIC TNM classifications. (Stage II vs. stage IIIC, $P < 0.01$.)

were surrounded by CD8- or CD4-positive cells. The number of mature DCs at the invasive margin was correlated significantly with that of CD8-positive T cells or that of CD4-positive T cells in the cancerous region. Patients with high numbers of CD8-positive or CD4-positive T cells in the cancerous region had significantly better prognoses than those without. The present study also showed that CD83-positive patients had a significantly lower incidence of lymph node metastases, suggesting a relationship between the number of mature DCs at the invasive margin and the likelihood of lymph node metastasis.

Taken together, the prognostic significance of CD83-positive DCs at the invasive margin may be explained as follows. When more abundant mature DCs are located at the invasive margin, there is a greater chance of T cells encountering mature DCs, and an increase in the number of activated T cells is expected, which might result in prevention of the spread of the cancer and a reduction in the incidence of lymph node metastases and, consequently, a better prognosis.

Various factors induce DC maturation. These include microorganisms,[23-25] presence of the CD40 li-

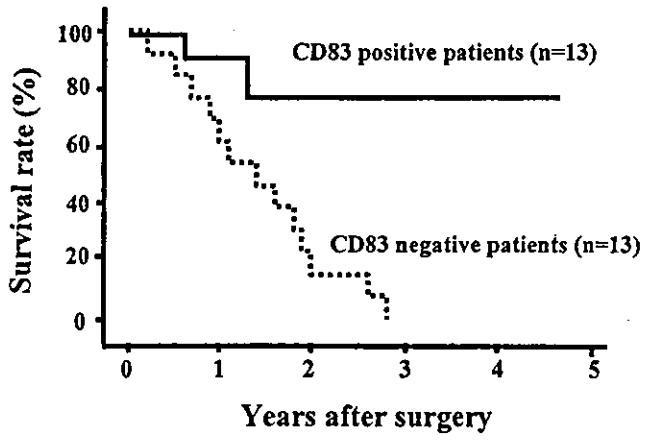

FIGURE 9. Five-year survival curves of CD83-positive (solid line) and CD83-negative (dotted line) patients (5-year survival rate, 78.6% vs 0%, respectively; $P < 0.001$).

gand on activated T cells,[26-28] cytokines (eg, tumor necrosis factor α, interleukin-1β), bacterial and viral products, [29-31] and nucleotides, but HSPs are the most likely candidates to explain this effect.[32] Surface expression of grp94 on tumor cells stimulates DC maturation in vitro and induces efficient T-cell priming and tumor rejection in vivo.[33] After maturation, DCs are no longer able to bind grp94 molecules; hence the grp94 receptor, CD91, is down-regulated.[34] Regression of some tumors, including melanoma and breast cancer, after injection with DCs has been observed, and associated with up-regulation of tumor HSP expression and infiltration of lymphocytes in response to HSP derived from autologous tumors.[35] In the present study, grp94 itself was not found to be a prognostic factor. In cancers immunohistochemically positive for grp94, however, a significantly higher number of CD83-positive DCs was observed at the invasive margin than those in grp94-negative cancers. This finding suggests that cancer grp94 expression is related to CD83-positive DCs in patients with CCC, but there is no relationship with CD1a-positive immature DCs.

In conclusion, mature DCs may influence the infiltration of CD8- and CD4-positive cells into CCC, with a consequent improvement in prognoses, due in part to abatement of lymph node metastasis.

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