However, the combined assay with α4GnT mRNA can detect pancreatic cancer patients more efficiently when compared with the enzyme immunoassays targeting single biomarkers.

The present study also demonstrated that the expression levels of α4GnT mRNA were elevated in 40% of chronic pancreatitis patients and 17% of cancer-free volunteers, albeit at much lower levels than those of pancreatic cancer patients. Previously, we showed that significant amounts α4GnT mRNA were detected in patients with *H. pylori* infection or chronic gastroduodenal ulcers.[17] It has been reported that unexpected genes such as α-fetoprotein are transcribed in lymphocytes when they are activated,[25] suggesting the possibility that α4GnT mRNA might be induced in the activated lymphocytes circulating in the *H. pylori*-infected patients. However, as shown previously[17] and further confirmed here, we have demonstrated that α4GnT mRNA is not detectable in activated lymphocytes or resting lymphocytes. By contrast, we have also reported that extensive biopsy of the gastric mucosa results in elevation of the α4GnT mRNA level in peripheral blood.[26] These results combined suggest that the gastric gland mucous cells expressing α4GnT mRNA enter the bloodstream through the injured sites of gastric mucosa caused by inflammation or biopsy. Considering the high incidence of *H. pylori* infection in individuals over 40 years of age in Japan,[27,28] α4GnT mRNA detected in the cancer-free volunteers is most likely derived from gastric gland mucous cells that have entered the peripheral blood through injured sites of the gastric mucosa caused by *H. pylori* infection. We previously demonstrated that α4GnT mRNA is not detected in the peripheral blood of healthy volunteers without *H. pylori* infection.[17] Similary, it may also be possible that α4GnT mRNA detected in the chronic pancreatitis patients originated from the α4GnT-positive pancreatic duct epithelia entering the blood circulation, because the disruption of pancreatic ducts could occur in chronic pancreatitis (Fig. 1c).[29] Further studies will be of significance to identify the cells that elevate the α4GnT mRNA level in the peripheral blood of these non-cancerous patients.

Recently we demonstrated that α4GnT is expressed not only in pancreatic carcinoma cells but also in biliary tract carcinoma cells that produce α1,4-GlcNAc-capped *O*-glycans.[15] Thus, the α4GnT assay will also be applicable to the detection of patients with biliary tract cancers. We have shown that α4GnT mRNA was detected in three of five patients with biliary tract cancer.[17] It is of great significance to determine the clinical utility of the α4GnT assay for diagnosis of biliary tract cancer as well.

Collectively, our results obtained in the present study indicate that quantitative analysis of α4GnT mRNA expressed in the peripheral blood allowed us to detect pancreatic cancer cells expressing α1,4-GlcNAc-capped *O*-glycans. In order to clarify the clinical contribution of this assay system, prospective controlled trials are needed in the screening, diagnosis and monitoring of pancreatic cancer patients.

Acknowledgments

This study was supported by Grant-in-Aids for 3rd Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor and Welfare of Japan and Scientific Research on Priority Areas 14030032 and 14082201 from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to JN), and by NIH Grant CA48737 from the National Institutes of Health (to MF). The authors thank Drs Tsutomu Katsuyama and Fukuto Maruta for encouragement during this work. We are also grateful to Dr Elise Lamar for critical reading of the manuscript.

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doi: 10.1111/j.1349-7006.2006.00148.x

□ ORIGINAL ARTICLE □

Ten Years of Experience with Liver Transplantation for Familial Amyloid Polyneuropathy in Japan: Outcomes of Living Donor Liver Transplantations

Yo-ichi TAKEI, Shu-ichi IKEDA, Toshihiko IKEGAMI*, Yasuhiko HASHIKURA*, Shin-ichi MIYAGAWA*, Yukio ANDO** and Japanese Liver Transplantation Society

Abstract

Object **We summarize 10 years of experience with liver transplantation for FAP patients in Japan and review the current opinions regarding this treatment for FAP.**

Methods and Patients **All basic report data on patients at the time of transplantation were registered with the Japanese Liver Transplantation Society (JLTS). Based on the JLST report data, more detailed information on FAP patients was requested from each center.**

Results **Living donor liver transplantation (LDLT) for FAP patients was first performed in Japan in 1993. LDLT has since been performed in 41 FAP patients, including nine cases of temporary auxiliary partial orthotopic liver transplantation (APOLT). Orthotopic liver transplantation (OLT) from cadaveric donors for FAP patients began in 1999, but only one FAP patient has subsequently undergone this procedure. Of these total of 43 FAP patients, 36 are currently alive: the one-year survival rate of patients after transplantation was 93%, and the five-year survival rate of these cases was 77%. Preoperative clinical severity and the nutritional status of patients are correlated with their outcome after liver transplantation. Domino (sequential) liver transplantation has been carried out in 20 domino recipients with end-stage liver diseases. Of the 20 domino recipients, 12 are currently alive.**

Conclusion **For FAP patients, these outcomes after the operation were very similar to those of OLT from cadaveric donors reported in other countries. Therefore, we concluded that for the treatment of FAP, LDLT from a living donor is equally effective as OLT from a cadaveric donor.**

(Internal Medicine 44: 1151–1156, 2005)

Key words: familial amyloid polyneuropathy, living donor liver transplantation, Japan

Introduction

Familial amyloid polyneuropathy (FAP) is one type of hereditary generalized amyloidosis, initially showing polyneuropathy and autonomic dysfunction but with later involvement of many visceral organs (1). Four endemic areas of this disease are known to exist in Portugal (2), Sweden (3), and Japan (1). The precursor protein of amyloid fibrils is a variant form of transthyretin (TTR) in serum (4). The vast majority of patients show the typical clinical picture known as type I FAP: the disease is caused by a mutation in the TTR gene resulting in the substitution of methionine for valine at position 30 (Val30Met) (5). Polyneuropathy begins in the legs and progresses in an ascending fashion. As TTR is produced mainly in the liver, liver transplantation for FAP patients was first performed in 1990 in Sweden (6), which was shown to result in the disappearance of variant TTR with Val30Met from the sera of transplant recipients. Since then, more than 800 patients in 16 countries have undergone orthotopic liver transplantation (OLT) (7). Moreover, domino (sequential) liver transplantation using a graft from the FAP patient was first performed in Portugal (8) in 1995, and more than 200 domino transplant procedures reutilizing explanted FAP livers had been performed by the end of 2002 (7, 9).

From the Third Department of Medicine, *the First Department of Surgery, Shinshu University School of Medicine, Matsumoto and **the Laboratory Medicine, Kumamoto University School of Medicine, Kumamoto

Received for publication February 16, 2005; Accepted for publication July 1, 2005

Reprint requests should be addressed to Dr. Yo-ichi Takei, the Third Department of Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621

The first liver transplant in a Japanese FAP patient was carried out in November 1993 at Shinshu University Hospital (10, 11), using living donor liver transplantation (LDLT). This transplantation method that includes domino liver transplantation has since been employed at different institutes in Japan. Here, we summarize 10 years of experience with liver transplantation for FAP patients in Japan and review the current opinions regarding this treatment for FAP.

Materials and Methods

All basic report data on patients at the time of transplantation were registered with the Japanese Liver Transplantation Society (JLTS). Centers report data on patients at the time of transplantation, operative procedures, including transplantation from living donor or cadaveric donor, and/or temporary auxiliary partial orthotopic liver transplantation (APOLT), and patient death. Based on the JLST report data, more detailed information on FAP patients was requested from each center: patients' weight, height, and serum albumin levels are necessary for calculation of the modified body mass index (mBMI). TTR gene mutation, neurological severity of FAP at transplantation, and pre- and postoperative states were also obtained. Furthermore, data regarding domino liver transplantation and Japanese FAP patients who underwent orthotopic liver transplantation (OLT) from a cadaveric donor overseas were collected.

Table 1. Liver Transplantation for FAP Patients in Japan (Nov. 1993–Jan. 2004)

City	Hospital	No. of transplants
Matsumoto	Shinshu University Hospital	24
Kumamoto	Kumamoto University	7
Nagoya	Nagoya University	4
Fukuoka	Kyushu University	2
Tokyo	Tokyo University	2
Kyoto	Kyoto University	1
Tokyo	Keio University	1
Sapporo	Hokkaido University	1
Niigata	Niigata University	1
	Total	43

Table 2. Number and Type of TTR Mutations

TTR mutation	Number
Val30Met	36
Tyr114Cys	3
Val30Leu	2
Ser50Ile	1
Ser50Arg	1

Statistical analysis

Student's *t*-test was used to determine the significance of differences between normally distributed means. Kaplan-Meier estimation for actual patient survival curves was performed. All statistical analyses were two-sided and were conducted at the 0.05 significance level. Data are given as means±SD.

Results

Liver transplantation for FAP

By January 2004, a total of 43 patients had undergone liver transplantation in 9 centers in Japan (Table 1). The patients consisted of 21 men and 22 women, ranging in age at liver transplantation from 26 to 63 years old (mean±SD, 40±9 years old). Disease duration before liver transplantation ranged from 0.5 to 10 years (mean±SD, 3.2±2.0 years). Five different TTR gene mutations were reported (Table 2). Among these, the Val30Met TTR mutation was the most common, including 36 cases. Seven had non-Val30Met TTR mutations: Tyr114Cys (n=3), Val30Leu (n=2), Ser50Ile (n=1), and Ser50Arg (n=1). Of the total of 43 patients, 41 had undergone LDLTs from living donors. Of these 41 LDLTs, APOLT was performed in 9 patients. Only 2 patients had undergone OLT from cadaveric donors.

Based on the neurological severity before transplantation, patients were staged clinically according to the criteria proposed by Coutinho et al (12): some autonomic dysfunction was seen in 4 patients without any sensory or motor neuropathy (stage 0); twenty-six patients had polyneuropathy localized to the lower limbs (stage I); and neurological dysfunction was in the more advanced stages in the remaining 13 patients (stages II or III).

Among the total of 43 patients, 36 were alive, including one patient who showed graft failure after APOLT and was treated by removal of the transplanted graft 1 week later. Twenty-seven patients returned to their previous normal lives after transplantation, and 9 were in hospital or recuperated at home. Seven patients died after transplantation: with regard to the severity of FAP, three patients were in stage I and 4 were in stage II, and their causes of death were as follows: infection (n=2), hepatic artery thrombosis (n=2), multiple cerebral infarction (n=1), heart failure (n=1), and uterine carcinoma (n=1). The registry analysis during the follow-up of more than 1 year showed that mBMI of patients who returned to their normal lives were significantly higher than those of patients in hospital or recuperating at home ($p<0.05$) (Fig. 1).

The one-year survival rate of all patients after transplantation was 93%, and their five-year survival rate was 77% (Fig. 2). The one-year survival rate of patients after LDLT was 93%, and their five-year survival rate was 76%.

Living donors of LDLT

Among the 43 transplantations, 41 were LDLTs. All of the donors had undergone TTR gene diagnosis and were

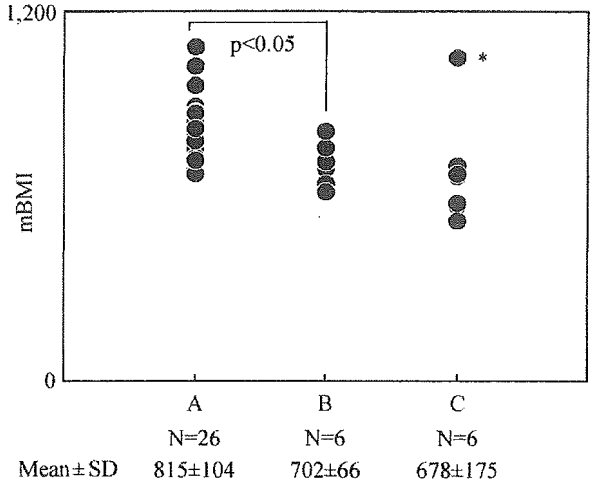

Figure 1. Relationship between outcomes after liver transplantation and modified body mass index (mBMI). A: Return to normal life after liver transplantation; B: Remain in hospital or recuperate at home (more than 1 year follow-up); C: Dead after liver transplantation. *: A patient who died from endometrial carcinoma after liver transplantation. Patients who returned to their normal lives had a significantly higher mBMI at the time of liver transplantation than those who remained in hospital or recuperated at home.

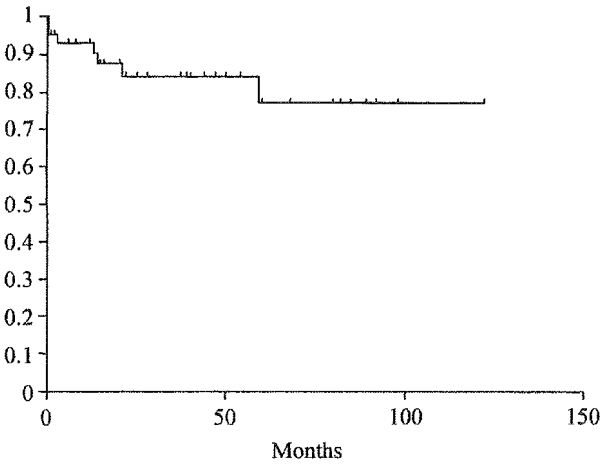

Figure 2. Patient survival after liver transplantation. The one-year survival rate of patients after transplantation was 93%, and their five-year survival rate was 77%.

shown to be negative for TTR gene mutation. The age at operation ranged from 19 to 66 years old (mean±SD, 46±11), and they consisted of 25 men and 16 women. The intrafamilial relationships between living donors and FAP patients are shown in Table 3. All of the living donors are alive and returned to their normal lives after the operation.

Table 3. Intra-familial Relationships between Living Donors and FAP Patients

Living donor	Number
Husband or wife	15
Siblings	11
Parents	10
Son or daughter	3
Nephew	1
Aunt	1

Table 4. Indications for Domino Recipients

	Number
Hepatocellular carcinoma	7
Primary sclerosing cholangitis	3
Congenital biliary atresia	2
Wilson's disease	2
Acute liver failure	1
Type II citrullinemia	1
Polycystic liver & kidney	1
Primary biliary cholangitis	1
Primary hyperoxaluria	1
Viral liver cirrhosis	1

Domino liver transplantation

Twenty recipients underwent domino liver transplantation using the livers of FAP patients since 1999 in Japan. The age at transplantation ranged from 17 to 64 years old (mean±SD, 43±14 years old). The indications of domino recipients are shown in Table 4. Among the 20 domino recipients, 12 were alive and 6 were dead, with no data available for the remaining 2.

Japanese FAP patients receiving OLT overseas

Twenty-one FAP patients with the Val30Met TTR mutation have undergone OLT from cadaveric donors (11 men and 10 women). Eight underwent OLT in Sweden, and 13 were treated in Australia. The ages at transplantation ranged from 28 to 49 years old (mean±SD, 36±6 years old). Disease duration before transplantation ranged from 1 to 6 years (mean±SD, 3.0±1.4). Among these 21 patients, only one died early after transplantation because of primary non-functional transplanted graft. The 20 surviving patients were all in good condition, and returned to their normal lives.

Discussion

Liver transplantation is now globally accepted for treatment of FAP patients. This therapy abolishes the hepatic source of amyloidogenic TTR variants in serum and progression of the disease has at least halted in the patients receiving

successful operations (13, 14). Approximately 65 to 70 transplantations per year are carried out on FAP patients worldwide (15). In Japan, there are two large foci of FAP patients (Arao city area in Kumamoto prefecture and Ogawa village area in Nagano prefecture), and there are many additional FAP families genetically unrelated to former endemic areas (16). Nevertheless, the number of FAP patients undergoing liver transplantation in Japan is relatively small, with only 3 to 4 transplantations per year being performed for Japanese FAP patients. One important reason is that OLT from cadaveric donors has not been widely accepted in Japan. Approximately 2000 transplantations from living donors have already been performed in Japan. However, based on the records of the JLST, until January 2004 the total number of OLTs from cadaveric donors in Japan is only 23, including the first case with Val30Met TTR type FAP treated successfully in 1999 (17). The graft size from a living donor is much smaller than that from a cadaveric donor, which results in long-term postoperative hospitalization for FAP patients. In addition, postoperative recovery is sometimes problematic in FAP patients receiving LDLTs (18). Despite these unfavorable conditions, the postoperative survival rate of FAP patients undergoing LDLT from living donors is very similar to that of patients undergoing OLT from cadaveric donors in other countries; the five-year survival rate of FAP patients undergoing LDLT in Japan was 76%, and according to The Familial Amyloidotic Polyneuropathy World Transplant Registry (FAPWTR) in Sweden, that after OLT was approximately 80%.

The prognosis of FAP patients after liver transplantation seems to be dependent on their preoperative state (19). FAPWTR emphasized that the severity of autonomic symptoms significantly affected the patient's recovery after operation, because many usually appeared in the patients at later stages of FAP (15). However, gastrointestinal autonomic symptoms, including severe episodic nausea, vomiting, and alternating constipation and diarrhea, are often initial symptoms in Japanese patients with this disease (1), and these bowel dysfunctions improved shortly after transplantation in the FAP patients in our series (11, 18). Therefore, FAP patients with only autonomic disturbances are considered good candidates for liver transplantation.

The mBMI is useful for estimating the nutritional status of patients. This index was reported to be correlated well with the prognosis of FAP patients after transplantation in foreign countries (20, 21). The mBMI of patients who returned to their normal lives after liver transplantation in our country were significantly higher than those in hospital or recuperating at home at follow-up after more than one year. The mBMI of patients who died after transplantation tended to be lower than those in patients with good outcomes, except in one patient who died of endometrial carcinoma 4 years 11 months after transplantation. Our results regarding mBMI support those of previous reports (20, 21), indicating that the nutritional state of FAP patients before transplantation is an important factor in predicting their post-operative state.

FAPWTR reported that the type of TTR gene mutation is a useful prognostic parameter (7, 15). A better 5-year patient survival was reported in Val30Met TTR type FAP patients, as compared to those with non-Val30Met TTR type FAP (80% *vs.* 59%, respectively, $p<0.001$). In Japan, 2 patients with Val30Leu TTR type FAP underwent LDLT, but both died approximately one year after the operations. Autopsy revealed heavy deposition of amyloid on the myocardium in these two patients (22). Serious cardiac involvement by amyloid is known to be one cardinal feature in FAP patients with non-Val30Met TTR type (16, 23), and this severe phenotype of cardiac amyloidosis has been suggested to be responsible for the poor post-transplant prognosis in these FAP patients. Recently, it was noted that after transplantation rapid deterioration of cardiac function with further thickening of the ventricular wall occurred in some FAP patients, although polyneuropathy and autonomic dysfunction were stabilized or slightly improved. These findings were originally obtained in non-Val30Met TTR type patients who may have had substantial amyloid deposition on the myocardium prior to the operation (24–26). However, a similar finding was reported in typical FAP patients with Val30Met TTR type (27). Wild-type TTR is regarded as playing a central role in the pathogenesis of this form of cardiac amyloidosis (22): wild-type TTR is inherently amyloidogenic, causing senile systemic amyloidosis (28), and conversion of normal TTR to the amyloid fibril conformation may be promoted after liver transplantation using a preexisting template of variant TTR-derived amyloid deposits in the myocardium. Therefore, it is critical to clarify the severity of cardiac amyloid deposition when considering liver transplantation in FAP patients. To overcome this problem, combined heart and liver transplantation was carried out in a small number of FAP patients in other countries (29).

As FAP patients who could obtain LDLT from a living donor in Japan were restricted, twenty-one FAP patients have undergone OLT in Australia or Sweden over the past 10 years (30). Their preoperative conditions of FAP were not serious, which enabled them to go abroad, and the resultant post-operative outcomes were satisfactory.

Liver tissue explanted from FAP patients has normal structure and function except for the production of amyloidogenic variant TTR, and domino liver transplantation using FAP liver grafts has been employed to compensate for the shortage of available liver grafts (7–9). In other countries, 70% of these operations were performed in patients with liver tumors, and 30% for non-malignant conditions (7). In Japan, 20 domino recipients with different liver disorders underwent liver transplantation using grafts from FAP patients, and 65% of them suffered from non-malignant diseases. Development of FAP symptoms has not been reported in any of the domino recipients at up to 7 years follow-up worldwide. However, Sousa et al reported that fibrillar deposits of TTR were observed in the epineurium of the biopsied peripheral nerve in a domino recipient with a 6-year post-operative history (31). Serial and careful follow-up of domino

recipients is necessary.

In conclusion, LDLT from a living donor for the treatment of FAP is equally effective as OLT from a cadaveric donor and has changed the natural course of patients with this disease (32, 33). Early intervention (less than 5 years after onset) can provide a better chance of improving patients' conditions after transplantation (34). Considering that the graft size from a living donor is much smaller than that from a cadaveric donor, earlier transplantation is desirable. However, this treatment cannot prevent the production of variant TTR in the retina (35) or choroid plexus (36), and long-term follow-up FAP recipients should undergo detailed examinations of ocular and central nervous system manifestations.

Acknowledgements: We would like to thank all reporting centers for their excellent work in reporting to the JLTS: Dr. Hiroyuki Furukawa (Hokkaido University Hospital), Dr. Motohide Shimazu, Dr. Masaki Kitajima (Keio University Hospital), Dr. Hideya Kamei (Nagoya University Hospital), Dr. Yoshihiko Maehara, Ms. Rie Nakao (Kyushu University Hospital), Dr. Katsuyoshi Hatakeyama (Niigata University Hospital), Dr. Masatoshi Makuuchi, Dr. Yuichi Matsui (Tokyo University Hospital), and Dr. Koji Umeshita (Osaka University, Graduate School of Medicine, Surgery and Clinical Oncology).

This work was supported by a grant from the Intractable Disease Division, the Ministry of Health and Welfare, Amyloidosis Research Committee, Japan; a Group Research Grant for the Pathogenesis and Therapy for Hereditary Neuropathy (10B-3).

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American Journal of Gastroenterology
© 2005 by Am. Coll. of Gastroenterology
Published by Blackwell Publishing

ISSN 0002-9270
doi: 10.1111/j.1572-0241.2005.00265.x

Expression of Fas Ligand by Hepatic Macrophages in Patients with Fulminant Hepatic Failure

Atsuyoshi Mita, M.D.,[1] Yasuhiko Hashikura, M.D.,[1] Yoh-ichi Tagawa, Ph.D.,[2] Jun Nakayama, M.D.,[3] Masatomo Kawakubo, Ph.D.,[3] and Shin-ichi Miyagawa, M.D.[1]

[1]*Department of Surgery, Shinshu University School of Medicine,* [2]*Department of Organ Regeneration, Shinshu University Graduate School of Medicine, and* [3]*Department of Pathology, Shinshu University School of Medicine, Matsumoto, Japan*

OBJECTIVES: The mechanisms of Fas–Fas ligand (Fas–FasL)-mediated apoptosis in the pathogenesis of fulminant hepatic failure (FHF) have not been well defined. This clinical study was carried out to assess which cells expressed Fas–FasL and to determine their involvement.

METHODS: The subjects were 24 patients with FHF who underwent liver transplantation at our institution. For comparison, nine chronic hepatitis (CH) patients and six living liver donors (LD) were also enrolled. Liver tissues were obtained for histological (hematoxylin–eosin, terminal deoxynucleotidyl transferase [TdT]-mediated dUTP-biotin nick-end labeling [TUNEL], immunohistochemistry, and double immunofluorescence staining) and reverse transcription PCR (RT-PCR; cytokines and chemokines) analysis.

RESULTS: The numbers of TUNEL-, FasL-, and CD68-positive cells in the livers of patients with FHF were significantly larger than in those with CH or with normal livers. Double immunofluorescence staining showed that FasL was expressed predominantly on liver macrophages and rarely on CD8-positive lymphocytes. RT-PCR study showed increased expression of FasL; interferon-γ; interleukin-18; macrophage inhibitory protein-1β; and regulated upon activation, normal T cell expressed and secreted in the livers of patients with FHF compared with those of LD.

CONCLUSIONS: Macrophages and their expression of FasL may play roles in the pathogenesis of FHF.

(Am J Gastroenterol 2005;100:2551–2559)

INTRODUCTION

The pathogenesis of fulminant hepatic failure (FHF) has not been profoundly defined, although several hypotheses, including those on the roles of tumor necrosis factor-α (TNF-α) (1), viral mutation (2), disturbance of the hepatic microcirculation (3), and apoptosis (4), have been presented and discussed. The significance of apoptosis via the Fas–Fas ligand (Fas–FasL) system has been the focus of studies in experimental models (5, 6) and in the clinical setting (7, 8). Intraperitoneal administration of agonistic anti-Fas antibody (Jo-1) in mice results in rapid killing with severe liver damage, as occurs in FHF (5). Hepatocyte apoptosis has been demonstrated in patients with FHF (4, 8), and although hepatocytes have been shown to express Fas (4, 7, 8) there have been no reports specifying which cells express FasL in the livers of patients with FHF. Although T-lymphocytes express FasL and play important roles in patients with chronic viral hepatitis (7, 9), there is a definite difference in the clinical features of patients with chronic hepatitis (CH) and those with FHF.

We verified the occurrence of hepatocyte apoptosis in the explanted livers of patients with FHF who underwent liver transplantation. In addition, we determined which cells (*i.e.*, macrophages and/or lymphocytes) expressed FasL. The aim of our clinical investigation was to elucidate the pathogenesis of FHF, with the ultimate aim of improving the treatment for this devastating condition.

METHODS

Patients

Our subjects were 24 patients with FHF who underwent living donor liver transplantation at Shinshu University between 1993 and 2003. Patient profiles are shown in Table 1. The definition of FHF was based on the findings of a previous report by Bernuau *et al.* (10), and the criteria adopted by King's College Hospital (11) were used as indications for liver transplantation. The causes of FHF were non-A, non-B, and non-C hepatitis in 16 patients, hepatitis B in 5, and idiosyncratic drug reaction in 3.

Liver Tissues

All tissues examined were obtained from explanted livers during liver transplantation. In addition, we examined liver tissues from nine patients with CH (liver tissue was obtained

Table 1. Profiles of Patients with Fulminant Hepatic Failure

Patient	Age	Gender	Etiology	Total Bilirubin (mg/dL)	Prothrombin Time (%)	Coma Grade
1	15 yr	M	Drug	17.8	16.0	4
2	4 yr	F	NANBNC	12.1	36.0	3
3	5 months	M	Hepatitis B	34.5	25.0	2
4	11 yr	M	NANBNC	11.8	40.0	2
5	6 yr	F	NANBNC	10.8	17.0	4
6	1 yr	F	NANBNC	6.4	7.0	3
7	18 yr	F	NANBNC	38.8	21.0	3
8	7 yr	M	NANBNC	24.5	33.0	2
9	36 yr	F	Drug	29.4	13.0	4
10	1 yr	M	NANBNC	13.9	12.0	3
11	6 months	M	NANBNC	9.3	18.0	4
12	23 yr	F	NANBNC	17.2	17.0	3
13	6 months	F	NANBNC	29.5	5.0	3
14	23 yr	F	Hepatitis B	10.3	6.7	4
15	1 yr	M	NANBNC	15.2	10.0	3
16	4 months	F	NANBNC	14.9	11.1	2
17	35 yr	F	NANBNC	16.9	13.0	4
18	44 yr	F	Hepatitis B	17.3	22.4	3
19	19 yr	M	NANBNC	28.2	12.0	3
20	9 yr	M	NANBNC	25.8	14.0	4
21	41 yr	M	Hepatitis B	24.9	27.0	4
22	20 yr	F	Hepatitis B	6.2	15.0	4
23	8 months	M	NANBNC	21.2	28.0	2
24	42 yr	F	Drug	9.0	40.6	3

NANBNC: non-A, non-B, and non-C hepatitis.

from noncancerous parts of the hepatectomized liver in cases of chronic hepatitis complicated by hepatocellular carcinoma, and informed consent for additional use of the samples was obtained) and from six living liver donors (LD) (liver tissue was obtained for histological assessment of the liver grafts and informed consent for additional use of the samples was obtained). The causes of the CH were hepatitis C in four, non-A, non-B, and non-C hepatitis in four, and hepatitis B in one. Informed consent was obtained from each patient, and the study was approved by the ethics committee of Shinshu University.

Each specimen was fixed in buffered formalin solution and embedded in paraffin wax. Serial sections (4–5 μm) were cut and mounted on clear silicon-coated glass slides (Dako, Tokyo, Japan). Ten specimens randomly selected from these 24 patients with FHF were obtained and were frozen in liquid nitrogen and stored at −80°C until use.

TUNEL Staining

To detect cells undergoing apoptosis, histological sections were stained by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) procedure, with some modifications as described previously (12, 13). We counted the number of TUNEL-positive cells in high-powered fields (×200) of liver tissues. The number of TUNEL-positive cells was calculated as the mean of 10 views of each liver tissue.

Immunohistochemistry

Immunohistochemical stainings for Fas, FasL, CD68, and CD8 were performed on serial hepatic tissue sections using a Dako LSAB kit (Dako). For antigen retrieval, microwave irradiation in 0.01-M citric acid buffer (pH 6.0) (for Fas and CD68) or Tris–citrate EDTA (pH 8.0) (for CD8) was carried out before application of the primary antibodies. The primary antisera used were mouse anti-Fas monoclonal antibody (ZB-4, MBL, Nagoya, Japan), rabbit anti-FasL polyclonal antibody (Nichirei, Tokyo, Japan), mouse anti-CD68 monoclonal antibody (KP1, Dako), and mouse anti-CD8 monoclonal antibody (C8/144B, Dako).

The histopathology of the liver was examined after hematoxylin and eosin staining, and the expression of each antigen was evaluated by counting the number of positive cells in high-power fields (×200) of the liver tissues. The number of positive cells was determined as the mean value of 10 views of each liver tissue specimen, with duplicate measurements.

Double Immunofluorescence Staining

Co-localization of FasL and other markers such as CD8, CD68, and hepatocytes was examined using double immunofluorescence staining methods. Mouse monoclonal antibody, OCH1E5, specific for hepatocytes was purchased from Dako. For antigen retrieval, we performed microwave irradiation in 0.01-M citric acid buffer (pH 6.0) (for FasL/CD68 as well as FasL/hepatocyte double staining) or Tris–citrate EDTA (pH 8.0) (for FasL/CD8 double staining). Tissue sections were reacted with each pair of primary antibodies, *i.e.*, FasL and CD68, FasL and CD8, and FasL and hepatocyte. Immunoreaction was detected by using a set of secondary antibodies: goat anti-rabbit IgG conjugated with rhodamine for FasL and goat anti-mouse IgG conjugated with FITC

for CD8, CD68, and hepatocyte antigen (Beckman Coulter, Tokyo, Japan).

The stained sections were mounted with Vectashield Hard Set mounting medium (Vector, Burlingame, CA, USA) and observed under an LSM 510 META laser scanning confocal microscope (Carl Zeiss, Tokyo, Japan). We calculated the number of positive cells in a sample as the mean of the counts in six fields of view (×200). Negative control experiments, in which the primary antibodies were replaced by the same host's serum, consistently showed no staining (data not shown).

RNA Extraction by Reverse Transcription PCR (RT-PCR)

Analysis of mRNA expression was performed in 10 randomly selected samples from patients with FHF and in two randomly selected samples from LD. Liver tissue was homogenized with a liquid-nitrogen-cooled mortar and pestle; the RNA was isolated by guanidium isothiocyanate and acid-phenol extraction (14). The RNA pellet was washed in 75% ethanol and resuspended in 20 μL of diethylpyrocarbonate treated with autoclaved Tris-citrate EDTA. RNA (2 μg) was reverse transcribed to cDNA by a SuperScript II first-strand synthesis system with oligo dT primer (Invitrogen Co., Carlsbad, CA, USA) and amplified using primers for human Fas; FasL; interferon-γ (IFN-γ); interferon-γ receptor α (IFN-γ Rα); interleukin-18 (IL-18); macrophage inhibitory protein 1β (MIP-1β); regulated upon activation, normal T cell expressed and secreted (RANTES); and β-actin (Table 2). PCR was performed, using Ex Taq DNA polymerase (Takara, Otsu, Japan) for 40 cycles, as follows: 94°C for 60 s (dissociation), 65°C (FasL) or 55°C (the others) for 30 s (annealing), and 72°C for 2 min (primer extension). Amplicons were visualized by 1.5% agarose gel electrophoresis.

Statistics

All data were expressed as means ± SEM. Statistical analyses were evaluated by Mann-Whitney's U-test using StatView software (SAS Institute Inc., Cary, NC, Version 5.0). Differences were designated as significant at $p < 0.05$.

RESULTS

Histological Characteristics of the FHF Liver

Although most hepatocytes had been lost and many hepatic lobules destroyed in the FHF liver, sinusoidal endothelial cells were present and dense mononuclear cells were observed (Fig. 1). In 15 of the 24 FHF patients, the remaining or regenerated hepatocytes were observed in islet formations that were sharply delimited by dense mononuclear cells. In the other 9 FHF patients, small numbers of hepatocytes were detected as pseudo bile ducts surrounded by many mononuclear cells. A large proportion of the mononuclear cells was lymphocytes and macrophages.

Apoptosis in the FHF Liver

To investigate whether apoptotic cells were present in the FHF liver, we examined DNA fragmentation as a marker of the presence of apoptotic cells by TUNEL staining of liver tissues obtained from the 24 FHF patients. Surviving hepatocytes were stained positively by TUNEL staining. The TUNEL-positive cells were present in the marginal zones of the surviving islets of hepatocytes (Fig. 2). The number of TUNEL-positive cells in the FHF liver was significantly greater than that in the CH liver or in the LD liver (Fig. 2).

Expression of Fas and FasL in Liver Tissue

The immunohistochemical studies revealed prominent staining for Fas on the surfaces and/or in the cytoplasm of hepatocytes in the FHF liver (Fig. 3). Lower levels of Fas staining were seen on the cell membranes and/or cytoplasm in the CH liver, and Fas staining in the LD liver was slight. Results of the Fas-positive hepatocyte count are shown in Figure 3.

Table 2. Genes and Primer Sequences in Reverse Transcription PCR Analysis

Gene	Primer Sequences	Amplification (bp)
Fas	5′CAG AAC TTG GAA GGC CTG CAT C3′	682
	5′TCT GTT CTG CTG TGT CTT GGA C3′	
FasL	5′GGA TTG GGC CTG GGG ATG TTT CA3′	344
	5′TTG TGG CTC AGG GGC AGG TTG TTG3′	
IFN-γ	5′GCA TCG TTT TGG GTT CTC TTG GCT GTT ACT GC3′	427
	5′CTC CTT TTT CGC TTC CCT GTT TTA GCT GCT GG3′	
IFN-γ Rα	5′GCT GTA TGC CGA GAT GGA AAA3′	588
	5′AGG AAA ATG GCT GGT ATG ACG3′	
IL-18	5′GCT TGA ATC TAA ATT ATC AGT C3′	342
	5′GAA GAT TCA AAT TGC ATC TTA T3′	
MIP-1β	5′ACC CTC CCA CCG CCT GCT GC3′	188
	5′GTT CCA GGT CAT ACA CGT ACT CC3′	
RANTES	5′ACC ACA CCC TGC TGC TTT GC3′	159
	5′CCG AAC CCA TTT CTT CTC TGG3′	
β-actin	5′ACT ACC TCA TGA AGA TCC TCA3′	441
	5′CAG GAG GAG CAA TGA TCT TGA3′	

FasL = Fas ligand; IFN-γ = interferon-γ; IFN-γ Rα = interferon-γ receptor α; IL-18 = interleukin-18; MIP-1β = macrophage inhibitory protein-1β; RANTES = regulated upon activation, normal T cell expressed and secreted.

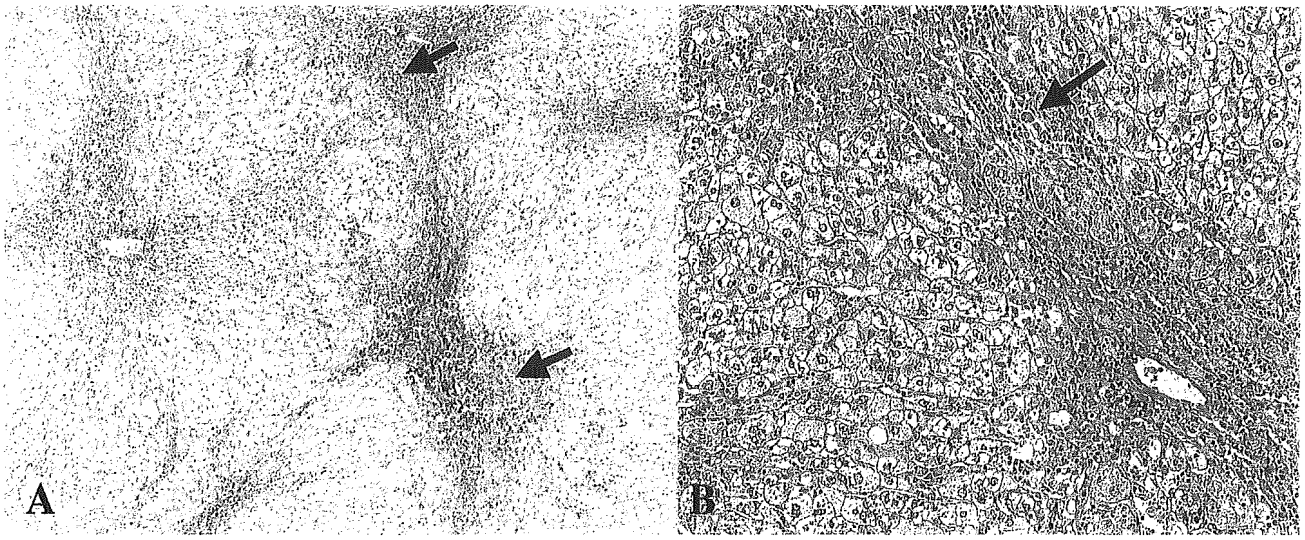

Figure 1. Representative histology of remaining hepatic lobules from a patient with FHF (Case 12). (*A*) Remaining hepatic lobules are composed of degenerating hepatocytes showing marked ballooning. The collapsed intervening parenchyma is visible among the lobules (arrows) (original magnification, ×20). With the orcein stain, the collapsed area contains no demonstrable elastic fibers. (*B*) Large numbers of inflammatory mononuclear cells surround the hepatic lobules (arrow). (Hematoxylin-eosin staining; original magnification, ×100)

FasL was stained predominantly on mononuclear cells around the margins of the hepatocyte islets (Fig. 4). The number of cells staining positively for FasL was significantly greater in the FHF liver than in the CH and LD livers.

Macrophage and Cytotoxic T-Cell Infiltration of FHF Liver

To identify the nature of the mononuclear cells, we immunostained the FHF liver tissues using anti-CD68 antibody as a marker of macrophages and anti-CD8 antibody as a marker of cytotoxic T cells. Enlarged CD68-positive cells were present around the damaged hepatocyte islets in the FHF liver (Fig. 5). Enlarged CD68-positive cells were present around the pseudolobules in the CH liver (data are not shown). In the LD liver, CD68-positive cells were observed only in the sinusoids. CD8-positive cells were found in similar locations in the FHF (Fig. 6) and CH livers. There was no significant difference in the count of CD8-positive cells between the FHF liver and the CH liver.

Cellular Localization of FasL

To characterize the cells expressing FasL in the FHF liver, we performed double immunofluorescence staining using CD68 or CD8 or anti-hepatocyte antibody and FasL (Fig. 7). There were significantly more CD68-positive cells co-expressing FasL in the FHF liver (41.71 ± 8.99/hpf) (Fig. 7A) than in the CH liver (0.79 ± 0.21/hpf) (Fig. 7C) ($p < 0.0002$). In contrast, there were significantly fewer CD8-positive cells co-expressing FasL in the FHF liver (1.18 ± 0.22/hpf) (Fig. 7B) than in the CH liver (6.32 ± 2.56/hpf) (Fig. 7D) ($p < 0.0001$). No hepatocytes expressed FasL (Fig. 7E).

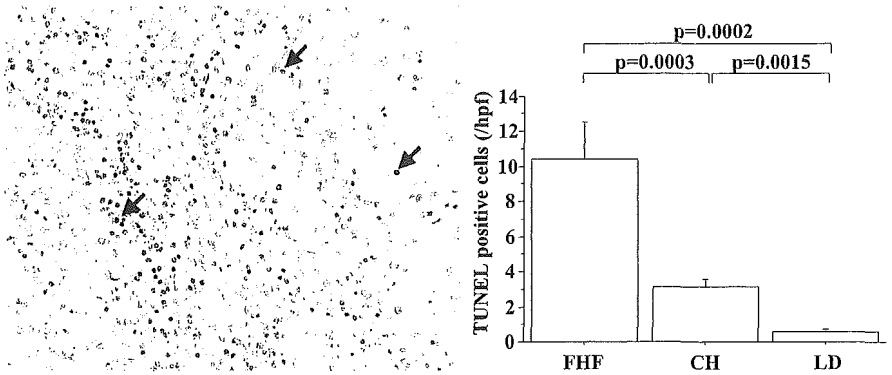

Figure 2. TUNEL staining of FHF liver tissue (Case 12). TUNEL-positive hepatocytes are found at the periphery of the remaining hepatic lobules (arrows), and inflammatory mononuclear cells being adjacent to them (original magnification, ×200). The numbers of TUNEL-positive cells were 10.45 ± 2.12/hpf, 2.77 ± 0.40/hpf, and 0.57 ± 0.14/hpf in the FHF, CH, and LD liver tissues, respectively.

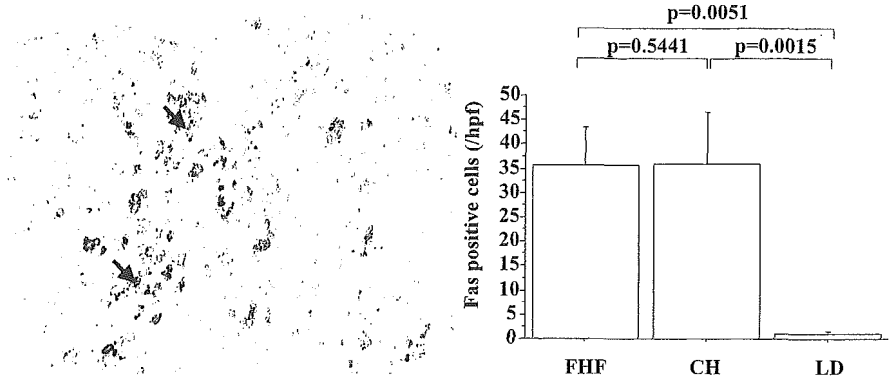

Figure 3. Representative immunohistochemistry of Fas in the FHF liver (Case 12). Fas is largely expressed by injured hepatocytes located in the marginal zones of the remaining hepatic lobules (arrows) (original magnification, ×200). Note that Fas is expressed in the cytoplasm of the hepatocytes. The numbers of Fas-positive cells were 35.76 ± 7.63/hpf, 23.22 ± 5.70/hpf, and 1.13 ± 0.38/hpf in the FHF, CH, and LD livers, respectively.

Semi-Quantitative Analysis of Cytokines and Chemokines in the FHF Liver

We performed semi-quantitative RT-PCR to evaluate mRNA expression of cytokines and chemokines in the FHF liver (Fig. 8). In the livers of seven patients with FHF, the mRNAs of FasL, IFN-γ, IL-18, MIP-1β, and RANTES were expressed more strongly than in the LD liver. Both the FHF liver and the LD liver showed expression of the mRNAs of Fas and IFN-γ Rα.

DISCUSSION

We verified the occurrence of hepatocyte apoptosis in the livers of FHF patients who underwent liver transplantation. In addition, we found that up-regulation of FasL occurred in most of the FHF liver and that the cells that expressed FasL were macrophages, not cytotoxic lymphocytes. Macrophages and their expression of FasL may play roles in the pathogenesis of FHF. These observations were different from those in the CH liver, especially in terms of the roles of cytotoxic lymphocytes. This is the first description of the cells in charge of FasL expression in FHF.

Although the pathogenesis of FHF has not been fully defined, the involvement of IFN and chemokines in the pathogenesis of FHF has been suggested in experimental models (15, 16) and in clinical studies (17, 18). Considering the potent proinflammatory functions of IFN, an unbalanced activation of the immune system caused by IFN plays a pivotal role in the pathogenesis of FHF (17). The main source of IFN is considered Kupffer cells, infiltrating macrophages, and lymphocytes (17). In addition, chemokines of the CC class are of particular interest in FHF, because they attract and activate macrophages and T-lymphocytes, the cellular hallmark of the intrahepatic infiltrates in this disease. Leifeld *et al.* (18) analyzed the role of CC-chemokines in the pathogenesis of FHF by examining serum levels and intrahepatic expression of monocyte chemoattractant protein-1, MIP-1α, MIP-1β, and RANTES in the livers and sera of patients with FHF and controls. Serum levels and intrahepatic expression of all chemokines studied in FHF exceeded the levels in chronic

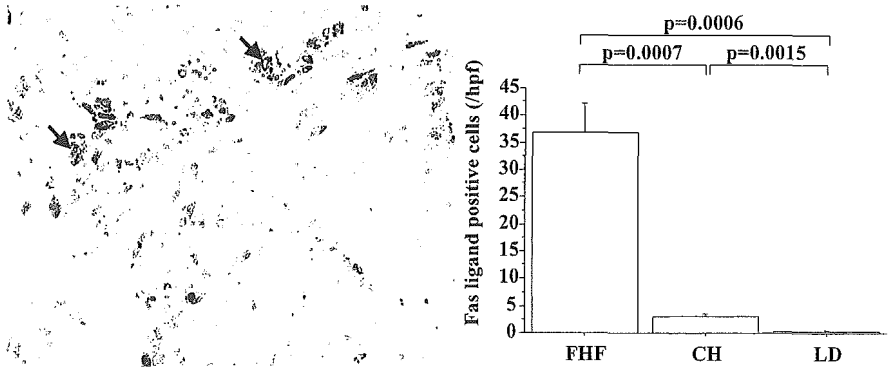

Figure 4. Representative immunohistochemistry of FasL in the FHF liver (Case 12). FasL is expressed by the inflammatory mononuclear cells infiltrating the marginal zones of the remaining hepatic lobules (arrows) (original magnification, ×200). Note that FasL is expressed in the entire cytoplasm of the mononuclear cells being adjacent to the injured hepatocytes. The numbers of FasL positive cells were 36.84 ± 5.30/hpf, 2.50 ± 0.55/hpf, and 0.30 ± 0.15/hpf in the FHF, CH, and LD liver tissues, respectively.

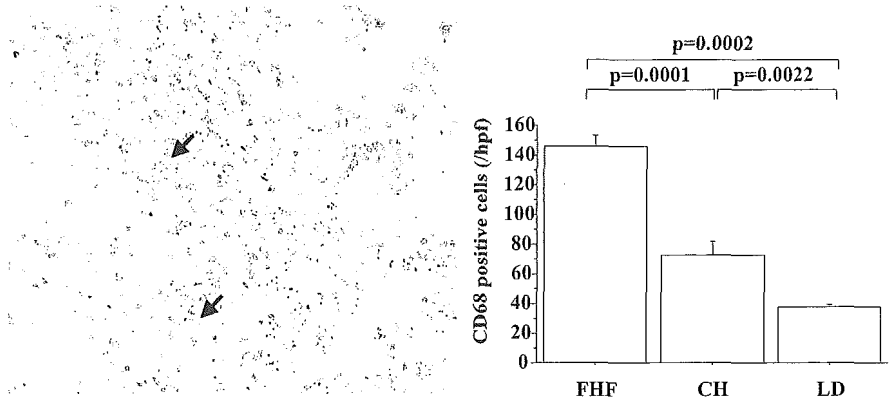

Figure 5. Representative immunohistochemistry of CD68 in the FHF liver (Case 18). CD68 is expressed in the macrophages infiltrating the remaining hepatic lobules (arrows) (original magnification, ×200).The numbers of CD68-positive cells were 145.68 ± 7.61/hpf, 60.45 ± 0.35/hpf, and 37.88 ± 1.61/hpf in the FHF, CH, and LD liver tissues, respectively.

liver diseases and normal controls (18). In fulminant hepatitis B, it has been indicated that massive induction of the proinflammatory cytokines IL-12 and IFN-γ is apparently not counterbalanced by the anti-inflammatory cytokine IL-10 (19). This cytokine imbalance may play an important role in promoting inflammatory reactions leading to massive liver damage in fulminant hepatitis B. Meanwhile, in chronic hepatitis C, Nischalke *et al.* (20) supported a correlation of the intrahepatic expression of the CC chemokine RANTES and the degree of periportal and portal inflammatory liver damage. The results of our RT-PCR study of liver tissue showed increased expression of FasL, IFN-γ, IL-18, MIP-1β, and RANTES in the FHF liver but not in the LD liver. These cytokines and chemokines may play roles in the pathogenesis of FHF. Hepatic macrophages have been shown to be responsible for IL-18 production in response to antigen stimulation (21). It has been suggested that activated hepatic macrophages produce IL-18 and then enhance IFN-γ production by CD8-positive T-lymphocytes, producing massive hepatic necrosis as well as virus elimination in FHF (22). IFN-γ has been shown to induce hepatocyte apoptosis through multiple pathways, including the Fas–FasL system, in an experimental setting using the Con-A hepatitis model in mice (23). Muschen *et al.* reported that administration of IFN-γ mediates FasL mRNA expression by rat Kupffer cells rather than by hepatic parenchymal cells or hepatic T-lymphocytes (24). RANTES has been shown to regulate the expression of FasL in HIV-specific cytotoxic T-cells followed by the killing of target cells by apoptosis (25). RANTES and MIP-1β expression leads to uncontrolled recruitment and activation of inflammatory cells—especially macrophages and cytotoxic lymphocytes—as an early step in the pathogenesis of FHF (18). In this clinical study, because of the ethical reasons, liver specimens were obtained at one point (at the time of liver transplantation) in the course of FHF. Taking into account the putative multistep pathogenesis as well as considerably redundant involvement of the chemokines in FHF, it is difficult to clearly assess the relevance of these cytokines and chemokines.

In terms of hepatocyte apoptosis in FHF, hepatocytes have been shown to express Fas, and the role of hepatocyte apoptosis that occurs via the Fas–FasL system has been the focus of some studies (6–9). However, no reports have specified which cells express FasL in the livers of patients with FHF.

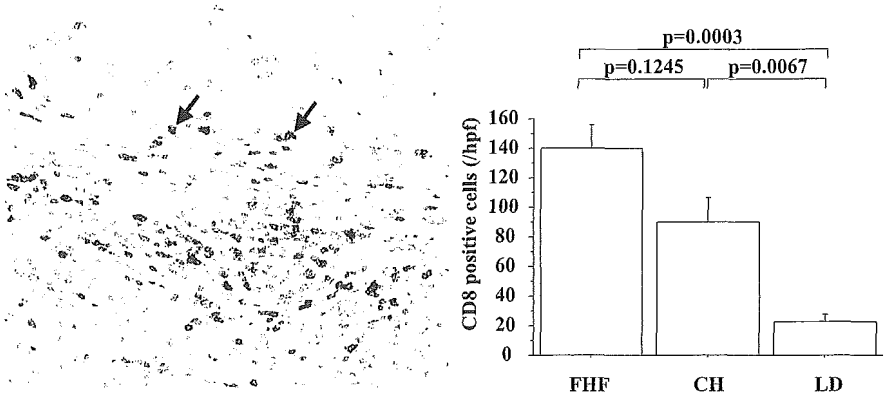

Figure 6. Immunohistochemistry of CD8 in the FHF liver (Case 9). Cytotoxic T cells positive for CD8 can be seen around the remaining hepatic lobules (arrows) (original magnification, ×200). The numbers of CD8-positive cells were 139.75 ± 16.22/hpf, 114.60 ± 15.70/hpf, and 22.62 ±5.04/hpf in the FHF, CH, and LD liver tissues, respectively.

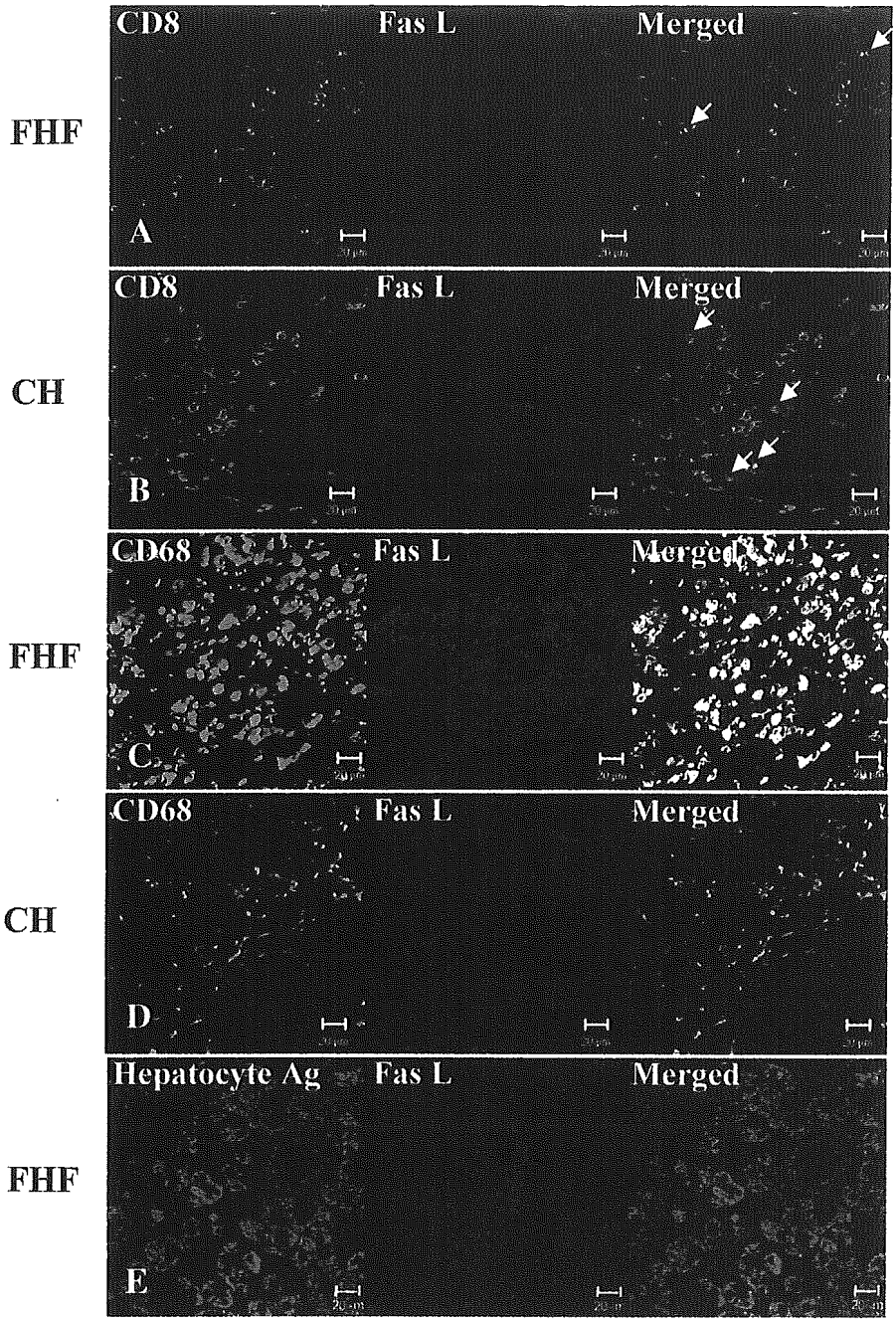

Figure 7. Double immunohistochemistry of FasL and other markers—CD8, CD68, and hepatocyte antigen—in FHF (Case 14) and CH (type B) livers. FasL-positive cells are indicated in red (middle column), and other markers are indicated in green (left column). Yellow coloration of the merged figures indicates double-positive cells (right column). (*A*) Cells double-positive for both FasL and CD8 were rarely detected in the FHF liver (arrows). (*B*) Cells double-positive for both FasL and CD8 were observed more frequently in the CH liver (arrows) than in the FHF liver. (*C*) Cells double-positive for both FasL and CD68 were observed frequently in the FHF liver. The small cells that are FasL+ and CD68− are red blood cells. (*D*) Few cells double-positive for both FasL and CD68 were detected in the CH liver. (*E*) FasL was not expressed in the hepatocytes, because cells positive for both FasL and hepatocyte antigen were not detected in the FHF liver.

We verified the occurrence of hepatocyte apoptosis in the explanted livers of patients with FHF who underwent liver transplantation and double immunofluorescence staining showed that FasL was expressed predominantly on liver macrophages. Fas is up-regulated in the hepatocytes of patients with various liver diseases, such as chronic hepatitis B (26), hepatitis C (27), alcoholic liver disease (28), Wilson's disease (29), and FHF (4, 7, 8). FasL has been shown to mediate hepatocyte apoptosis in mainly the initial stage of development of viral hepatitis (30). The fact that the localizations of TUNEL-positive cells, Fas-positive hepatocytes, and FasL-positive macrophages were similar, strongly suggests that

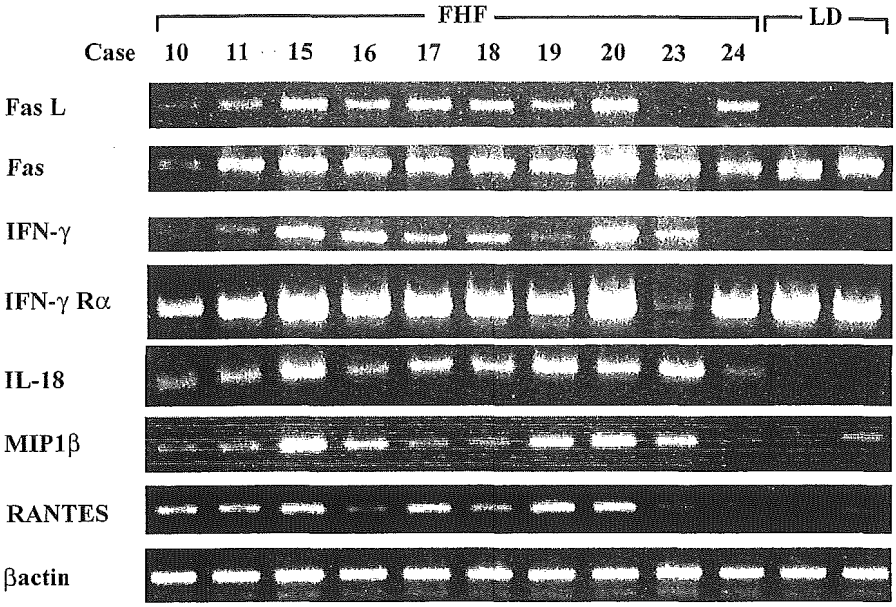

Figure 8. Semi-quantitative RT-PCR of cytokine and chemokine in the FHF and LD liver. The mRNA of the receptors such as Fas and IFN-γ Rα was expressed both in the FHF and LD liver. In contrast, mRNAs of Fas L, IFN-γ, IL-18, MIP-1β, and RANTES were expressed strongly in seven cases with FHF compared with the LD liver.

Fas–FasL-mediated apoptosis occurred in the FHF liver. FasL was expressed predominantly on liver macrophages but rarely on CD8-positive lymphocytes. These findings were different from those of CH livers, in which FasL was expressed mainly on CD8-positive lymphocytes and rarely on hepatic macrophages. Our observations in the CH liver accord with those of previous reports of CH in humans and in experimental liver injury (7, 9, 30–32). The increased number of macrophages but not of cytotoxic lymphocytes in the FHF liver compared with the CH liver is notable. This clinical finding is important for identifying the pathological difference between FHF and CH. In CH group, when compared between hepatitis C subgroup and non-A, non-B, and non-C subgroups, the above described findings were similar. Several reports have described the significance of FasL-positive liver macrophages in apoptosis of cells lining the intrahepatic bile ducts in primary biliary cirrhosis (33) and in hepatocyte apoptosis in hepatitis B (32). Ryo *et al.* reported FasL mRNA expression by lymphocytes in the liver in FHF (8), but our results did not confirm their finding.

Considering that activation of infiltration macrophages to the liver play pivotal roles in the pathogenesis of FHF (*e.g.*, mediating immune-mediated liver cell damage, chemokine network, and apoptosis), further investigation to establish a strategy to get macrophages under control during FHF needs to be formulated.

In summary, this clinical study suggests that hepatic macrophages play a role in the pathogenesis of FHF via FasL expression. Although the disease no doubt has a multifaceted pathogenesis, down-regulation of activated hepatic macrophages could be a useful strategy in the treatment of human FHF.

Reprint requests and correspondence: Yasuhiko Hashikura, M.D., Department of Surgery, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan.
Received January 7, 2005; accepted June 6, 2005.

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Original Paper

Interrelationship of Platelet-Derived Endothelial Cell Growth Factor, Liver Macrophages, and Tumor Microvessel Density in Patients with Cholangiocellular Carcinoma

Shiro Miwa MD, Junpei Soeda MD, Shin-ichi Miyagawa MD
First Department of Surgery, Shinshu University School of Medicine, Nagano, Japan
Corresponding Author: Shiro Miwa MD, Shinshu University School of Medicine
Asahi 3-1-1, Matsumoto, Nagano 390-8621, Japan
Tel: +81 263 37 2654, Fax: +81 263 35 1282, E-mail: smiwa@hsp.md.shinshu-u.ac.jp

KEY WORDS:
Cholangiocellular carcinoma; Platelet-derived endothelial cell growth factor; Macrophage; Microvessel density

ABBREVIATIONS:
Microvessel Density (MVD); Platelet-Derived Endothelial Cell Growth Factor (PD-ECGF); Vascular Endothelial Cell Growth Factor (VEGF); Basic Fibroblast Growth Factor (bFGF); Epidermal Growth Factor (EGF); Tumor Necrosis Factor Alpha (TNF-α)

ABSTRACT

Background/Aims: Microvessel density (MVD) has been studied extensively as the only factor reflecting angiogenesis and a prognostic factor in various malignant tumors. Macrophages and platelet-derived endothelial cell growth factor (PD-ECGF) also play important roles in regulating angiogenesis. The present study was conducted to examine the interrelationship of MVD, liver macrophages and PD-ECGF-positive cells in patients with cholangiocellular carcinoma.

Methodology: Thirty-one patients underwent resection of cholangiocellular carcinoma, and samples of the tumors were immunostained with CD34 antibody to evaluate the relationship between MVD and prognosis. Double immunohistochemical labeling for CD68 and PD-ECGF-positive cells was performed and classified as grade 0, grade 1, or grade 2 according to the number of double-positive cells. We also evaluated the relationship between the double-positive cell grading and prognosis or MVD, and furthermore the relationship between cancer cell PD-ECGF immunoreactivity and prognosis or MVD.

Results: Univariate analysis showed that patients with a median MVD exceeding 48/field had a significantly poorer prognosis ($p=0.02$). The survival rate of grade 2 patients was significantly worse than that of the other two groups ($p=0.011$, $p=0.0001$), and the survival rate of grade 1 patients was significantly worse than that of grade 0 patients ($p=0.007$). MVD differed significantly among the three grades ($p=0.0007$, Kruskal-Wallis test), and there was a significant positive correlation between MVD and grade ($p=0.0001$). No correlation was observed between MVD and the number of cells positive for PD-ECGF alone ($p=0.42$). Neither the survival rate nor MVD of PD-ECGF (+) patients differed significantly from that of PD-ECGF (-) patients ($p=0.08$, $p=0.6$).

Conclusions: Although the present results are based on a small number of patients, they suggest that liver macrophages at the invasive margin of cholangiocellular carcinoma might contribute to tumor angiogenesis through PD-ECGF secretion, and thus influence the prognosis of patients.

INTRODUCTION

Cholangiocellular carcinoma is now recognized to be the second most common primary liver cancer. It shows invasive and rapid growth, and is usually at an advanced stage by the time of diagnosis. Recently, it was reported that vascular invasion and lymph node metastasis are clinicopathological prognostic factors of cholangiocellular carcinoma (1-3).

Among various quantitative studies of tumor vascularity and neovascularization conducted using immunohistochemical staining methods (4), microvessel density (MVD) has received close attention as the only factor reflecting angiogenesis and a prognostic factor in various malignant tumors. Until now, there has been no reported analysis of MVD and prognosis in patients with cholangiocellular carcinoma. Several factors are known to stimulate angiogenesis, including vascular endothelial cell growth factor (VEGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), tumor necrosis factor alpha (TNF-α), and platelet-derived growth factor (PDGF). Macrophages are known to secrete more than 100 cell products which include the above angiogenic factors (5,6), suggesting that macrophages have an important role in regulating angiogenesis. The functions of macrophages, including Kupffer cells, in the liver are the same as those of macrophages at other sites. However, no reports have yet addressed the relationship between tumor vascularization and liver macrophages in cholangiocellular carcinoma.

Thymidine phosphorylase (TP), also known as platelet-derived endothelial cell growth factor (PD-ECGF), is an enzyme that has been implicated in tumor angiogenesis. Experimental studies have

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revealed that the expression of PD-ECGF in tumor cells is significantly correlated with intratumoral microvessel density (7,8) and some articles have reported an association between the expression of PD-ECGF in tumor cells and the overall survival of patients with malignant tumors (9,10). PD-ECGF expression in stromal cells, comprising fibroblasts, lymphocytes, macrophages, endothelial cells, smooth muscle cells, and Schwann cells, within cancer tissues, but not in the cancer cells themselves, has an important role in tumor angiogenesis and prognosis (11-14). Up to now, no reports have described the prognostic significance of the interrelationship of cell PD-ECGF expression, liver macrophages and angiogenesis in cholangiocellular carcinoma.

In the present study, therefore, we analyzed the interrelationship of MVD, liver macrophages and PD-ECGF positive cells immunohistochemically in 31 patients with cholangiocellular carcinoma, and assessed the prognostic significance of these factors.

METHODOLOGY

Between October 1989 and March 2002, 31 patients underwent surgical resection of cholangiocellular carcinoma at the First Department of Surgery, Shinshu University Hospital, leaving no macroscopic evidence of residual cancer. There was no operative or hospital death. After discharge, all the patients were followed up at our outpatient clinic on a monthly or bimonthly basis. The median follow-up time was 28 months (1-143 months).

Formalin-fixed paraffin-embedded specimens of tissues resected from these 31 patients were subjected to immunostaining. Permission was obtained preoperatively from each patient to use part of the resected cancer lesion for research.

Immunohistochemical Analysis of MVD

The surgically resected tissue samples, which had been fixed in 10% formalin and embedded in paraffin using the routine method, were cut into 4-μm-thick sections and mounted on glass slides coated with poly-L-lysine. Tissue samples were available from all patients and were suitable for evaluation of vessels by staining the endothelial cells for CD34. Three sections from one paraffin block per tumor were stained for CD34. Deparaffinized rehydrated tissues were incubated in 3% hydrogen peroxide for 30 minutes to block any endogenous peroxidase activity. The sections were then incubated at room temperature for 1 hour with an anti-human mouse monoclonal CD34 antibody (QBEhd/10) (NeoMarkers, Inc. Fremont, CA) at a dilution of 1:400 in 1% BSA. Antibody binding was visualized using the labeled streptavidin-biotin method (LSAB kit; Dako) and 3,3'-diaminobenzidine, and cell nuclei were counterstained with hematoxylin.

Double Immunohistochemical Labeling of Macrophages and PD-ECGF-positive Cells

Liver macrophages visualized by staining the antigen recognized by PG-M1 (an anti-CD68 monoclonal antibody), were evaluated. For staining of PD-ECGF, sections were incubated at room temperature for 1 hour with an anti-human monoclonal PD-ECGF antibody (NeoMarkers, Inc., CA) at a dilution of 1:200 in 1% BSA as the first primary antibody. Antibody binding was visualized using the labeled streptavidin-biotin method (LSAB kit; Dako) and 3,3'-diaminobenzidine. After retrieval by microwave heating, the sections were incubated at room temperature for 1 hour with an anti-human mouse monoclonal CD68 antibody (PG-M1) (Dako Co., Carpenteria, CA) at a dilution of 1:200 in 1% BSA as the second primary antibody. Antibody binding was visualized using the labeled streptavidin-biotin method (LSAB-ALP kit; Dako). For negative controls, all reagents were used except for the primary antibody.

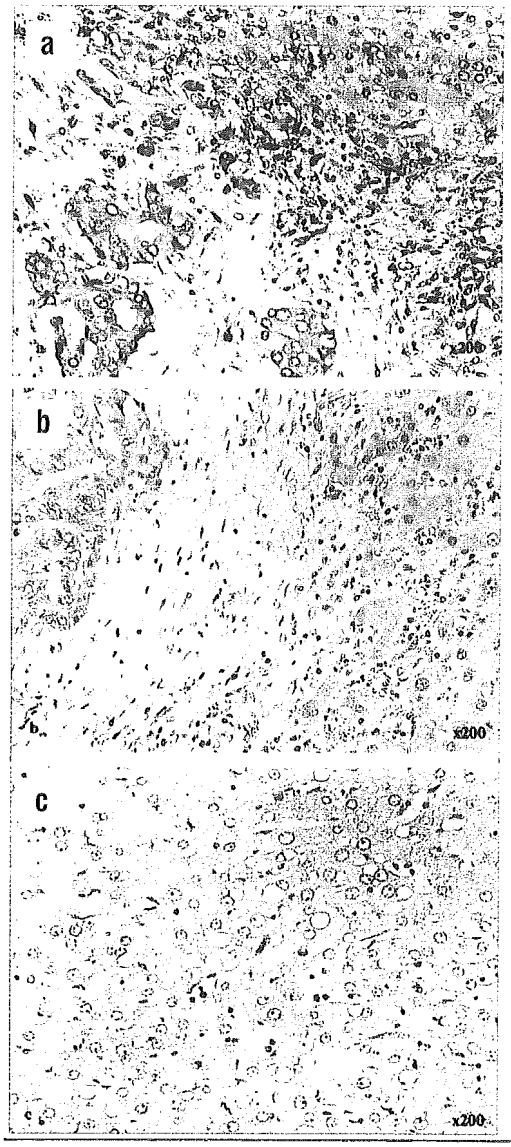

FIGURE 1
Immunostaining of PD-ECGF (brown) and PG-M1 (red).
(a) Immunoreactivity of PD-ECGF and PG-M1 at the invasive margin. Almost all PG-M1-positive cells showed positivity for PD-ECGF.
(b) Lack of immunoreactivity for PD-ECGF and PG-M1 at the invasive margin. Grade 0 patients had no PD-ECGF-positive cells.
(c) Immunoreactivity of liver distant from the tumor. Liver macrophages show spreading and adhesion to sinusoids, and no PD-ECGF-positive cells are observed.

Evaluation of MVD and Immunoreactivity of PG-M1-positive and PD-ECGF-positive Cells

Screening of the tumor was performed using a

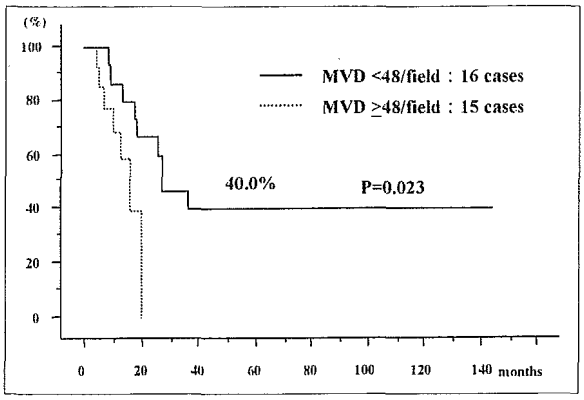

FIGURE 2 Survival curves for patients with a median MVD of <48/field and >48/field. Patients with a MVD of >48/field had a poorer prognosis than those with a MVD of <48/field (p=0.02).

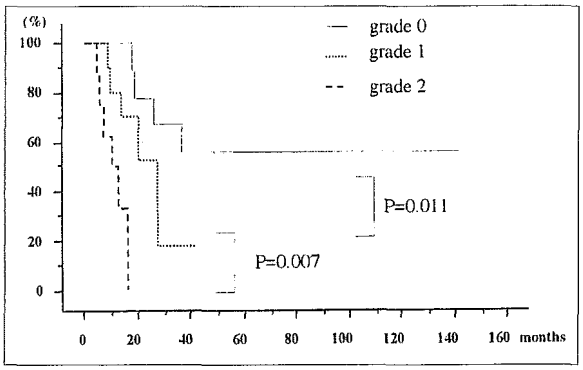

FIGURE 3 Survival curves for patients divided into 3 groups according to the number of CD68-positive cells. Survival rate of grade 2 patients was significantly worse than that of the other two groups (p=0.011, p=0.0001), and the survival rate of grade 1 patients was significantly worse than that of grade 0 patients (p=0.007).

light microscope equipped with x10 objective lens and x10 ocular lens (Olympus, Tokyo, Japan) to identify the three areas of highest neovascularization in the tumor. Color (RGB) images of the area on CD-34-stained sections at x200 magnification were captured by a Fujix digital camera HC 2500 (Fuji Photo Film (Co., Ltd.), Tokyo, Japan) connected to a Macintosh personal computer (Apple Computers, Cupertino, CA) running the Adobe PhotoShop program (Adobe Systems Incorporated, San Jose, CA). Microvessels were evaluated and counted according to Weidner *et al.* (4) simultaneously by two investigators without knowledge of the patient's outcome. Both observers agreed that any positive cell or cell cluster, which was clearly separate from adjacent microvessels, tumor cells, and other connective tissue elements, was a single, countable microvessel.

Double staining of PG-M1 (red) and PD-ECGF (brown) demonstrated that almost all CD68-positive cells were also positive for PD-ECGF **(Figure 1a)**. In high-power fields (x400), the numbers of interstitial and infiltrating cells positive for both PG-M1 and PD-ECGF were counted at the cancer invasive margin. In 10 patients, no double-positive cells were observed at the invasive margin (categorized as grade 0) **(Figure 1b)**. In the remaining 21 patients, the three areas showing the highest count of infiltrating cells at the invasive margin were identified, and the ratio of double-positive cells, relative to all interstitial and infiltrating cells was evaluated. These 21 patients were then categorized as either grade 1 (median double-positive cell ratio <35) or grade 2 (>35%).

The immunohistochemical staining for PD-ECGF-positive cancer cells was evaluated by two independent observers in a blind manner, and the entire area of each section was observed. Immunoreactivity of cancer cells for PD-ECGF was classified as negative (-) if <5% of the total number of cancer cells were immunopositive, and as positive (+) if >5% of the cancer cells were immunopositive.

Statistical Analysis

Data are reported as means ± SD. We used Kruskal-Wallis one-way analysis of variance on ranks to compare overall differences among the three groups. We compared MVD in all groups using the Mann-Whitney U test with Bonferroni correction. Because two pairwise planned comparisons were made, we considered differences at $p<0.025$ to be significant. When correlations were examined, the Spearman rank correlation coefficient was calculated. The survival curves were calculated by the Kaplan-Meier method and the statistical significance was compared by the log-rank test. Differences at $p<0.05$ were considered statistically significant. All analysis was performed with the Statview 5.0 statistical software package (Abacus Concepts, Berkley, CA).

RESULTS

CD34-positive cells or vessels were easily distinguished from surrounding tissues around the tumor border. Away from the tumor, anti-CD34 staining was confined to vessels of the portal triad, and sinusoids showed no reactivity. The area of highest vascularization was usually the tumor stroma between the tumor nests, and most prominently at the invasive margin. The mean number of CD34-positive cells or vessels in the tumor was 48±28.8/field. Univariate analysis showed that patients with a median MVD greater than 48/field had significantly poorer prognosis (p=0.02) **(Figure 2)**.

Double labeling for PD-ECGF and PG-M1 revealed that liver macrophages spread and adhered to sinusoids in non-cancerous regions, and that PD-ECGF-positive cells were absent in non-cancerous regions **(Figure 1c)**. When patients were divided into the 3 groups described above according to the count of double-positive cells, the survival rate of grade 2 patients was significantly worse than that of the other two groups (p=0.011, p=0.0001), and the survival rate of grade 1 patients was significantly worse than that of grade 0 patients (p=0.007) **(Figure 3)**. Furthermore, MVD differed significantly among the three groups (p=0.0007, Kruskal-Wallis test). The multiple comparison test showed that MVD differed significantly between grade 0 and grade 1 patients (33±17.6, 46±23.1, p=0.007), and between grade 1 and grade 2 patients (46±23.1, 64±27.3, p=0.020) **(Figure 4)**. A significant positive correlation was observed between