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A Decrease in AFP Level Related to Administration of Interferon in Patients with Chronic Hepatitis C and a High Level of AFP

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It is known that there is a very high incidence of hepatocellular carcinoma (HCC) among patients with type C chronic hepatitis and cirrhosis, and α-fetoprotein (AFP) has been widely used as a diagnostic marker for HCC. However, there are some patients showing continuous high AFP values but no evidence of HCC, and some studies have defined such patients as a high-risk group for HCC. In vitro study has shown that interferon (IFN) inhibits cell proliferation and enhances apoptosis as well as specific cytotoxic T lymphocytes against HCC, resulting in direct anticancer actions. In this study, we investigated the effect of IFN on AFP changes in chronic hepatitis C patients. Of 40 patients with chronic hepatitis C in whom diagnostic imaging confirmed the absence of HCC, 24 patients showed high pretreatment AFP values (high AFP group: AFP level > 10 ng/dl; mean ± SD, 46.3 ± 41.5 ng/dl) and 16 showed low pretreatment AFP values (low AFP group: pretreatment AFP level ≤ 10 ng/dl; mean ± SD, 5.3 ± 2.2 ng/dl). Pretreatment clinical parameters were statistically evaluated in relation to the AFP value. In the high AFP group, the platelet count, albumin level, and prothrombin (%) were significantly lower ($P = 0.047$, $P = 0.0002$, and $P = 0.044$, respectively), suggesting that AFP value increases with advancing liver disease. Subsequently 27 patients were administered IFN (IFN group), and the remaining 13 patients were administered Stronger Neo-minophagen C (SNMC), a glycyrrhizin preparation (SNMC group), as a control group receiving liver-protective therapy. Alanine aminotransferase was reduced in both the IFN and the SNMC group (mean, 132.56 to 60.07 mg/ml [$P < 0001$] and 147.85 to 56.23 mg/ml [$P = 0.0240$], respectively). AFP was significantly reduced in the IFN group (mean, 30.03 to 12.65 ng/ml; $P = 0.0034$), but there was no significant change in AFP in the SNMC group (mean, 29.70 to 39.17 ng/ml). AFP is useful for diagnosing HCC; however, some patients show a persistently high AFP level in the absence of HCC, and these patients have been described as a high-risk group for HCC. In this study, we found that IFN therapy but not SNMC universally reduced the AFP baseline. Since AFP is a significant predictor for HCC, therapeutic strategies for hepatitis C, e.g., long-term low-dose IFN treatment, may reduce hepatocarcinogenesis.

KEY WORDS: hepatitis C; interferons; hepatocellular carcinoma; α-fetoprotein.

Recently, combination therapy with pegylated interferon (IFN) and ribavirin for 48 weeks has achieved viral eradication in 54 to 56% of patients, and the occurrence of hepatocellular carcinoma (HCC) was prevented in these responders (1, 2). For nonresponders to IFN therapy, liver-protective therapy, such as oral administration of

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ursodeoxycholic acid or intravenous injection of Stronger Neo-minophagen C (SNMC), is commonly performed in Japan, and it is considered that these treatments may delay the progression of liver disease (3, 4). SNMC is a glycyrrhizin preparation that exhibits potent anti-inflammatory actions and has been used to treat allergic diseases and hepatitis in Japan for centuries. However, this agent is not considered to have any antiviral or anticancer ability (5), while IFN is considered to have antiviral, anti-inflammatory, and anticancer effects, and is employed in clinical practice to treat certain types of cancer, such as germ cell tumor and RCC (6, 7).

α-Fetoprotein (AFP) has been widely used as a diagnostic marker for HCC. However, there are some patients with a high AFP baseline but no evidence of HCC, although some papers have reported that AFP is a significant predictor of HCC in such patients (8, 9). This study investigated the clinical characteristics of such patients with a high AFP baseline and assessed the effect of IFN administration in terms of AFP changes, since AFP is suggested to be an important risk factor for HCC.

METHODS

Forty patients with type C chronic hepatitis and compensatory liver cirrhosis patients who were being followed at Kurume University Medical Center were retrospectively investigated. All patients were confirmed to be positive for serum hepatitis C virus (HCV)-RNA by polymerase chain reaction (PCR). HBs-Ag-positive, autoimmune, alcoholic, and drug-induced hepatitis patients were excluded from the study. Furthermore, the absence of HCC was confirmed by abdominal ultrasonography (US) or dynamic computed tomography (CT) in all subjects.

According to the pretreatment AFP value, the 40 subjects were divided into two groups: the high AFP group (AFP > 10 ng/dl; $n = 24$) and the low AFP group (AFP ≤ 10 ng/dl; $n = 16$). Then the pretreatment clinical background parameters were statistically investigated using the Mann-Whitney U-test and chi-square test to compare the high and low AFP groups.

These 40 subjects were divided into two groups, the IFN group ($n = 27$) and the SNMC group ($n = 13$). Six million units of recombinant IFNα-2b was injected intramuscularly three times a week or more in the IFN group. SNMC was administered intravenously three times a week at a dose of 40 to 100 ml in the SNMC group. Both alanine aminotransferase (ALT) and AFP values after 4 weeks of treatment were compared with the pretreatment values. Paired t-test was used, and $P < 0.05$ was regarded as significant.

RESULTS

Clinical Characteristics in Patients with High AFP Baseline (High AFP) vs. Low AFP Group. There were no significant differences in age, gender, ALT level, HCV genotype, or HCV-RNA level between the high and the low AFP groups; however, in the high AFP group, the platelet count, albumin level, and prothrombin (PT) value were significantly lower ($P = 0.0014$, $P = 0.0026$, and $P = 0.0041$) (Table 1). These results suggest that the AFP level increases with the progression of liver disease.

Pretreatment Backgrounds in IFN and SNMC Treatment Groups. There were no significant differences in the pretreatment background parameters such as AFP value, age, gender, ALT value, platelet count, albumin level, PT (%), and HCV-RNA level between the two groups (Table 2). Fourteen of the 27 IFN-treated patients (52%) showed a high pretreatment AFP value (>10 ng/ml), and 9 of the 13 SNMC-treated patients (69%) showed a high pretreatment AFP value (>10 ng/ml).

ALT Changes in IFN and SNMC Treatment Groups. With respect to changes in the ALT level, the AFP level was significantly decreased in the IFN group (132.6 ± 72.7 to 61.1 ± 43.3 U/L; $n = 27$; $P < 0.0001$). In the SNMC group, ALT levels were also significantly decreased (149.4 ± 17.2 to 83.0 ± 57.7 U/L; $n = 12$; $P = 0.019$) (Figure 1).

AFP Changes in IFN and SNMC Treatment Groups. As for AFP changes, the AFP value was significantly

TABLE 1. PRETREATMENT CLINICAL CHARACTERISTICS ACCORDING TO AFP VALUE

	High AFP (n = *24*) *(AFP > 10 ng/ml)*	*Low AFP* (n = *16*) *(AFP ≤ 10 ng/ml)*	P *value*
AFP (ng/ml)	46.264 ± 41.534	5.348 ± 2.229	—
Age (yr)	55.875 ± 9.252	52.938 ± 12.179	0.3914
Gender (M/F)	14/10	12/4	0.2790
ALT (U/L)	144.333 ± 88.122	125.813 ± 83.818	0.5108
PLT ($\times 10^4/\mu l$)	11.421 ± 4.997	14.550 ± 4.030	0.0467*
Albumin (g/dl)	3.617 ± 0.444	4.138 ± 0.238	0.0002*
PT (%)	72.368 ± 11.923	80.237 ± 10.796	0.0439*
HCV-RNA (KIU/mL)	472.667 ± 286.404	463.067 ± 323.334	0.9257

Note. Mann-Whitney U-test or chi-square test was used. $P < 0.05$ was considered significant.
Values are expressed as mean ± SD.

TABLE 2. PRETREATMENT PATIENT PROFILES IN THE SNMC AND IFN GROUPS

	SNMC (n = *13*)	*IFN* (n = *27*)	P *value*
AFP (ng/ml)	29.970 ± 35.229	30.030 ± 39.643	0.9798
Age (yr)	54.308 ± 10.427	54.889 ± 10.685	0.8719
Gender (M/F)	9/4	17/10	0.6071
ALT (U/L)	147.846 ± 110.816	132.556 ± 272.702	0.6039
Platelets (×104/μl)	11.015 ± 6.244	13.441 ± 3.870	0.1387
Albumin (g/dl)	3.738 ± 0.568	3.867 ± 0.408	0.4185
PT (%)	72.615 ± 13.775	77.615 ± 10.887	0.2607
HCV-RNA (KIU/mL)	502.900 ± 299.403	455.500 ± 302.124	0.6752

Note. Mann-Whitney *U*-test or chi-square test was used. $P < 0.05$ was considered significant.
Values are expressed as mean ± SD.

decreased in the IFN group (53.0 ± 44.3 to 20.3 ± 26.7 ng/ml; $n = 14$; $P = 0.0023$). Interestingly, all 27 IFN-treated patients showed a decrease in AFP value regardless of response to treatment. However, there was no significant change in the AFP value after SNMC administration (31.1 ± 36.4 to 39.0 ± 46.5 ng/ml; $n = 9$; $P = 0.11$) (Figure 2). Mean AFP value was slightly increased in the SNMC group.

DISCUSSION

AFP is a fetal protein that is not normally present in the serum of adults and is commonly used as a tumor marker for HCC. However, serum AFP is also elevated during pregnancy and in chronic hepatitis patients (10, 11). In this study, a considerable number of type C chronic hepatitis and compensated cirrhosis patients demonstrated persistently elevated AFP levels in the absence of HCC. In addition, the AFP level decreased significantly after IFN administration. Furthermore, the AFP decrement was universally observed regardless of treatment response to IFN therapy. Transient AFP elevation has been observed after a rise in transaminase in acute hepatitis and fulminant hepatitis (12–14). This type of AFP elevation is explained as a result of hepatocyte regeneration accompanied by necroinflammatory change. In this study, AFP was not changed in the SNMC group despite significant improvement in transaminase, suggesting that the AFP elevation was not caused by hepatocyte regeneration in chronic hepatitis patients.

AFP production is supposed to regulate the transcription level of hepatocytes (15). Among HCV-infected patients, the HCV-coding core protein is regarded to be one of the proteins responsible for hepatocarcinogenesis, up-regulating several molecules resulting in activation of the cell cycle and cell proliferation at the transcriptional level in hepatocytes (16). The HCV-coding core protein may also upregulate AFP production at the transcriptional

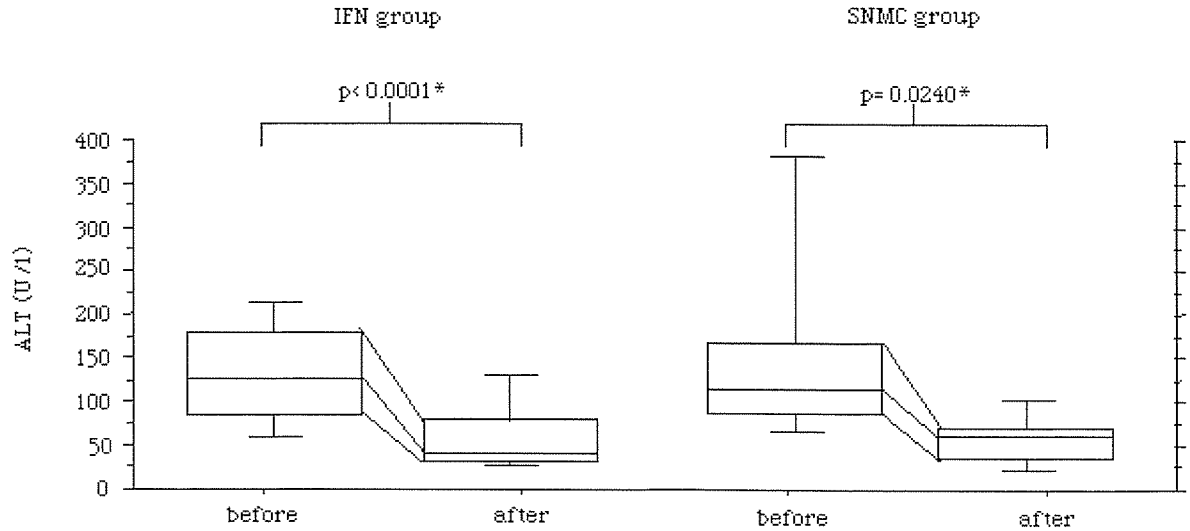

Fig 1. Changes in alanine aminotransferase (ALT) after IFN and SNMC administration. Paired *t*-test was used. *$P < 0.05$ was regarded as significant.

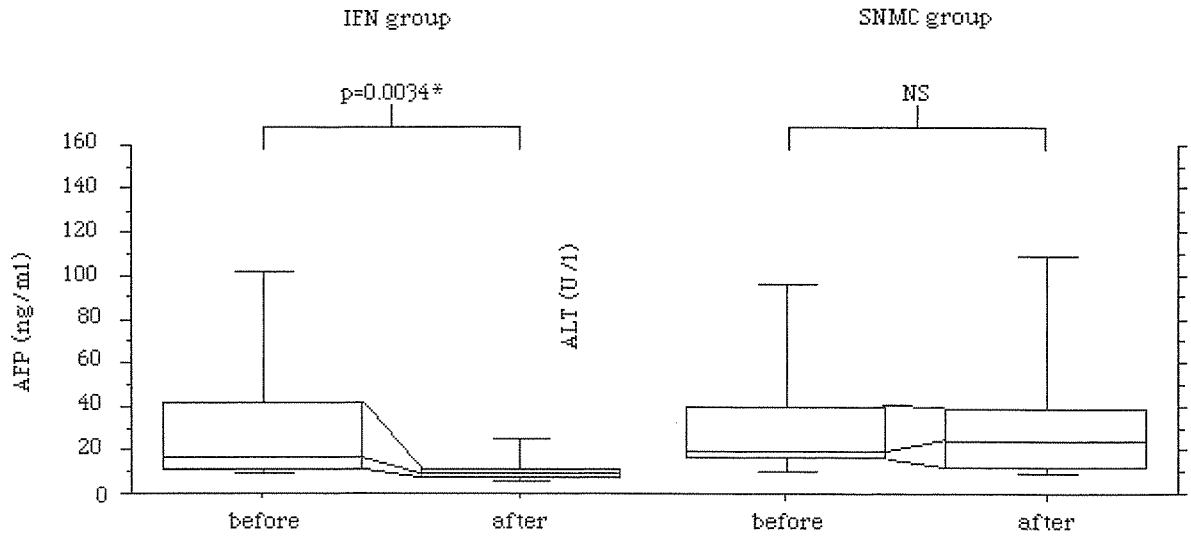

Fig 2. α-Fetoprotein (AFP) changes with IFN and SNMC administration Paired *t*-test was used. $*P < 0.05$ was regarded as significant. NS, not significant.

level. In contrast, IFN is considered to down-regulate cell cycle progression at the transcriptional level and induce apoptosis via the IFN receptor-mediated JAK-STAT signaling pathway (17). This competing action of IFN against HCV-related protein may be a direct anticancer mechanism that inhibits HCC. Actually, a clinical study has demonstrated anticancer effects of IFN administration against intrahepatic recurrence after resection of HCC (18), and IFN has also been used to treat HCC in combination with anticancer agents such as 5-fluorouracil (19).

Many reports have cited elevated AFP baselines as an independent HCC risk factor (8, 9) along with age, gender, liver histology stage, and ethnicity in HCV-infected patients. In the present study, the AFP baseline was decreased in all IFN-treated patients, even IFN nonresponders. This indicates that IFN therapy, rather than liver-protective therapy, universally reduces the risk factors of HCC in HCC high-risk subjects with high AFP values and advanced liver disease. Therefore, therapeutic strategies, such as long-term administration of low-dose IFN, may inhibit HCC in patients who have failed to respond to routine IFN treatment. Further investigation is needed to evaluate IFN effect in relation to AFP production and hepatocarcinogenesis.

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Endothelial Progenitor Cell Transplantation Improves the Survival Following Liver Injury in Mice

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Background & Aims: Neovascularization, which is vital to the healing of injured tissues, recently has been found to include both angiogenesis, which involves in mature endothelial cells, and vasculogenesis, involving endothelial progenitor cells. The aim of this study was to clarify the possible roles of endothelial progenitor cells during postnatal liver regeneration. *Methods:* To determine how endothelial progenitor cells participate in liver regeneration, human or mouse endothelial progenitor cells were transplanted into the mice with carbon tetrachloride-induced acute liver injury. Survival rate of the mice in endothelial progenitor cell-transplanted and control groups was calculated. Separately, livers removed temporally from both groups were examined. *Results:* At an early stage, transplanted human endothelial progenitor cells were seen mainly surrounding hepatic central veins where hepatocytes showed extensive necrosis; later, the transplanted cells formed tubular structures. More of these cells were observed along hepatic sinusoids. Transplantation of human or mouse endothelial progenitor cells improved survival of the mice following liver injury (from 28.6% to 85.7%, $P < .0005$ and from 33.3% to 80.0%, $P < .001$, respectively), accompanied by greater proliferation of hepatocytes. Human endothelial progenitor cells produced several growth factors, such as hepatocyte growth factor, transforming growth factor-α, heparin-binding epidermal growth factor–like growth factor, and vascular endothelial growth factor, and also elicited endogenous growth factors. *Conclusions:* Endogenous and exogenous growth factors and direct neovascularization after endothelial progenitor cell transplantation promoted liver regeneration, thus improving survival after liver injury. Transplantation of endothelial progenitor cells could represent a new therapeutic strategy for promoting liver regeneration.

Neovascularization is critical for the healing of injured tissues as well as proliferation of carcinoma cells in vivo because both processes require a supply of growth factors, nutrients, and oxygen.[1,2] Vasculogenesis, originally defined as vascularization during embryogenesis from endothelial progenitor cells (EPC) or angioblasts, begins as formation of blood islands composed of EPC at the periphery and hematopoietic stem cells in the center. In distinction, postnatal neovascularization has been believed to result from proliferation, migration, and remodeling of fully differentiated endothelial cells derived from preexisting blood vessels, a process referred to as angiogenesis. However, EPC recently were isolated from human peripheral blood and were shown to be incorporated into active angiogenesis sites.[3] Accordingly, the concept of postnatal neovascularization was revised to include both angiogenesis by mature endothelial cells and vasculogenesis by EPC.

The therapeutic potential of EPC populations expanded ex vivo recently has been investigated in rodent models of ischemic disorders that require neovascularization, such as myocardial and hindlimb ischemia.[4–7] Transplanted EPC proved to preserve cardiac ventricular function and to salvage limbs from necrosis in association

Abbreviations used in this paper: EPC, endothelial progenitor cell; Flk-1, fetal liver kinase-1; Flt-1, fms-like tyrosine kinase 1; HB-EGF, heparin-binding epidermal growth factor–like growth factor; HGF, hepatocyte growth factor; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MNC, mononuclear cell; PCNA, proliferating cell nuclear antigen; RT-PCR, reverse-transcriptase polymerase chain reaction; SEC, sinusoidal endothelial cell; TGF-α, transforming growth factor-α; Tie-2, tyrosine kinase with Ig and EGF homology domains-2; VEGF, vascular endothelial growth factor.

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with enhanced capillary density, supporting the therapeutic applicability of EPC transplantation to various ischemic diseases.[8]

In liver regeneration, sinusoidal endothelial cells (SEC), the resident endothelial cells, have been believed to proliferate, migrate, and reconstruct hepatic sinusoids.[9–14] In a recent study, bone marrow-derived cells, presumed to be EPC and monocyte-lineage cells, were found to contribute to hepatic sinusoid reconstruction in liver regeneration after partial hepatectomy in mice.[15] However, data are lacking concerning the role of EPC in liver regeneration.

The aims of this study were to obtain direct evidence of vasculogenesis in liver regeneration using isolated EPC to elucidate how EPC take part in liver regeneration and to explore the possibility of therapeutic application of vasculogenesis in liver disease.

Materials and Methods

Animals

Male Balb/c wild- and nude (immunologic unresponsiveness of genetically thymusless)-type mice[16] weighing 20 to 22 g (Japan SCC, Shizuoka, Japan) were used in all experiments. Mice were housed at a controlled temperature (22°C) under 12 h/12 h light-dark conditions and were maintained on a standard diet with freely available water. All mouse experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Kurume Institutional Animal Care and Use Committee.

Isolation of Mononuclear Cells

To obtain EPC, mononuclear cells (MNC) were isolated from peripheral blood of healthy human volunteers or bone marrow of mice by density gradient centrifugation with Histopaque-1077 (Sigma Chemical, St. Louis, MO). Cells were collected, washed twice with 10 mmol/L phosphate-buffered saline (PBS; pH 6.8), suspended into cell culture medium described below, and plated on gelatin (Sigma)-coated culture dishes. Informed consent was obtained from all volunteers. The study protocol was conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the Kurume University Human Research Committee.

Culture of Endothelial Progenitor Cell

Medium 199 supplemented with 20% fetal bovine serum, bovine pituitary extract to stimulate cell growth, heparin (100 μg/mL), and antibiotics (Sigma) was used for cell culture. Attached cells thus were allowed to develop into EPC until day 10 of culture when culture dishes were washed with PBS for immunocytochemistry or 1 mmol/L EDTA in PBS for EPC collection. EPC attached to dishes were treated with trypsin-EDTA solution (Sigma) for 3 minutes, gently scraped free, and collected. After washing with PBS, EPC were suspended in PBS at a density of 10^5/100 μL and injected into mice as described below.

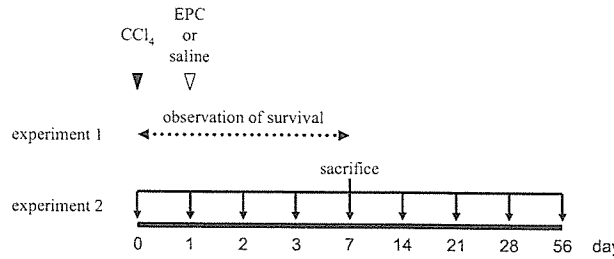

Figure 1. Summary of experimental procedure. Nude type mice received an administration of carbon tetrachloride intraperitoneally (day 0) and PBC with or without EPC via the spleen (day 1). Some mice were observed until day 7 to calculate the survival rate and the others were killed temporally to perform several experiments.

Cell Labeling

In experiments to detect EPC, these cells were labeled with PKH26GL (red fluorescent cell linker kit for general cell membrane labeling, Sigma) following the manufacturer's instructions before EPC transplantation to mice. In brief, EPC were collected from culture dishes and incubated in a solution containing PKH26 red fluorescent dye (2 μmol/L/mL) at room temperature for 5 minutes. After washing in PBS, EPC were transplanted into mice.

Liver Injury Model in Mice

The experimental procedure is summarized in Figure 1. To induce chemical liver injury, 200 μL/kg of carbon tetrachloride (CCl_4) diluted 10% (vol/vol) with olive oil was administered intraperitoneally into the nude-type mice. Mice received an injection of 100 μL of PBS with or without 10^5 EPC of human or mouse into the spleen 1 day after CCl_4 injection. Survival rate in the mice receiving an injection of PBS with or without human or mouse EPC was calculated until day 7 after CCl_4 injection. In separate experiments, the mice were killed at 0, 1, 2, 3, 7, 14, 21, 28, and 56 days after CCl_4 injection to obtain several tissues. Livers were perfused by PBS and processed for several experiments.

Immunocytochemistry

EPC on culture dishes were fixed in 4% paraformaldehyde diluted with PBS at 4°C for 20 minutes or in equal volumes of acetone and ethanol at −20°C for 10 minutes. To block nonspecific binding of primary antibodies, dishes were preincubated with Protein Block Serum-Free (DAKO, Kyoto, Japan) for 30 minutes. Primary antibodies are listed in Table 1. As primary antibodies in immunocytochemistry, antihepatocyte growth factor (HGF),[17] anti-transforming growth factor-α (TGF-α) (CALBIOCHEM, San Diego, CA), anti-CD34 antibody (DAKO), PE-conjugated anti-CD133 (Miltenvi Biotec GmbH, Bergisch, Gladbach, Germany), antifetal liver kinase-1 (Flk-1), anti-fms-like tyrosine kinase-1 (Flt-1), tyrosine

Table 1. Primary Antibodies

Antigen	Species
HGF	Rabbit
TGF-α	Rabbit
HB-EGF	Goat
VEGF	Rabbit
Flk-1	Rabbit
Flt-1	Rabbit
Tie-2	Rabbit
PCNA	Rabbit
CD31	Mouse
CD34	Mouse
CD133 (PE-conjugated)	Mouse

kinase with Ig and EGF homology domains-2 (Tie-2), antiheparin-binding epidermal growth factor-like growth factor (HB-EGF), and antivascular endothelial growth factor (VEGF) antibodies (Santa Cruz Biotechnology, Palo Alto, CA) were used. Dishes were incubated at 4°C overnight with primary antibodies diluted 1:100 (vol/vol) with PBS. Dishes were incubated for 60 minutes with FITC-conjugated anti-mouse, anti-rabbit, or anti-goat IgG antibody (Cappel, Aurora, OH) diluted 1:100 (vol/vol) with PBS. Finally, to protect fluorescence staining, specimens were embedded in Vectorshield (Vector Laboratories, Burlingame, CA).

Immunohistochemistry in formalin-fixed, paraffin-embedded sections. Conventionally processed and embedded sections cut at a thickness of 3 μm were deparaffinized with xylene and immersed in ethanol for 15 seconds. To block endogenous peroxidase activity, sections were then incubated in methanol with 3% H_2O_2 (vol/vol) for 10 minutes during deparaffinization. To perform immunostaining for proliferating cell nuclear antigen (PCNA), some sections then were autoclaved in 10 mmol/L citrate buffer at 121°C for 5 minutes and cooled slowly to room temperature. After washing in PBS with 0.05% Tween 20 (Wako, Osaka, Japan) (PBS-T), sections were preincubated with Protein Blocking Serum-Free (DAKO). Sections were primarily incubated at 4°C overnight with anti-PCNA, VEGF, or Tie-2 antibody (Santa Cruz), diluted 1:100 (vol/vol) with PBS. After washing 3 times at 4°C for 5, 10, and 15 minutes in PBS-T, sections were incubated with anti-rabbit Enivision Plus (DAKO) for 60 minutes. After washing again in PBS-T, sections were incubated in a solution containing 0.1% (wt/vol) 3-3′-diaminobenzidine-tetrahydrochloride and 0.005% (vol/vol) H_2O_2 in 0.1 mol/L Tris-HCl buffer (pH 7.6) for 3 minutes. Nuclear counterstaining was performed with Mayer's hematoxylin.

Immunohistochemistry in frozen sections. Frozen liver tissue sections were cut at a thickness of 6 μm. Sections were fixed in equal volumes of acetone and ethanol, washed in water, and then immersed in PBS-T. To perform immunohistochemistry in mouse liver with mouse primary antibody, following DAKO's instructions, the anti-CD31 antibody (DAKO) and FITC-conjugated anti-mouse IgG antibody (Cappel) diluted 1:100 (vol/vol) with PBS first were incubated together for 60 minutes. To prevent reaction of excess secondary antibody to endogenous mouse IgG, the excess secondary antibody was saturated 20:1 (vol/vol) with mouse serum (DAKO) for 60 minutes. After incubation of specimens with blocking regent, they were incubated at 4°C overnight with the immunecomplex described above or with Flk-1 antibody (Santa Cruz) diluted 1:100 (vol/vol) with PBS. Sections incubated with anti-Flk-1 antibody were allowed to react with FITC-conjugated anti-rabbit IgG antibody (Cappel). Sections were embedded in Vectorshield (Vector Laboratories).

Immunohistochemistry, enzyme-linked immunosorbent assay. Liver tissues were homogenized 1:4 (wt/vol) in a solution containing 20 mmol/L Tris-HCl buffer (pH 7.5), 2 mmol/L NaCl, 1 mmol/L PMSF, 1 mmol/L EDTA, and 0.1% Tween-20. After centrifugation, the supernatant was collected for enzyme-linked immunosorbent assay (ELISA). ELISA specific for mouse HGF was performed by SRL (Tokyo, Japan). Liver samples were obtained from 6 mice each in EPC-transplanted and control groups.

Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from liver tissues and EPC using Isogen (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions. RNA concentration was determined spectrophotometrically at 260/280 nm. Reverse transcription (RT) was performed to synthesize cDNA, using total RNA, random primers, deoxynucleotide triphosphates, and SuperScript III reverse transcriptase (Invitrogen Life Technology, Carlsbad, CA). The RNA and primers were mixed and denatured by heating at 70°C for 10 minutes. Next, the RT reaction mixture was incubated for 30 minutes at 50°C, followed by 15 minutes at 70°C. A no-template control was performed for each experiment, establishing the absence of genomic contamination of samples. Polymerase chain reaction (PCR) was performed for 30 cycles to amplify cDNAs encoding growth factors, using sense and antisense primers (Table 1), RT products as a template, AmpliTaq DNA polymerase (PE Applied Biosystems, Foster City, CA), and deoxynucleotide triphosphates (GIBCO, Rockville, MD) in a GeneAmp 9700 Sequence Detection System (PE Applied Biosystems). Denaturation, annealing, and primer extension, respectively, were performed at 94°C for 30 seconds, at primer-specific temperatures for 1 minute (Table 2), and 72°C for 1 minute. PCR products were analyzed on 1.5% ethidium bromide-stained agarose gels.

SYBR Green Real-time Quantitative PCR Assay

Real-time semiquantitative PCR was carried out using a GeneAmp 5700 Sequence Detection System (PE Applied Biosystems). The primers were designed to distinguish between mouse and human VEGF mRNA (Table 3).

Quantitative SYBR Green real-time PCR was performed using sense and antisense primers (Table 3), RT products, and SYBR Green PCR Core Reagents kit (PE Biosystems, Warrington, UK), according to the manufacturer's instructions.

Table 2. RT-PCR Primer Sequence for Several Growth Factors

Primer	Sequence (5′-3′)	Tann (°C)
HGF	5′-Primer: GCC TGA AAG ATA TCC CGA CA	66
	3′-Primer: TTC CAT GTT CTT GTC CCA CA	
HB-EGF	5′-Primer: AAA AGA AAG AAG AAA GGC AA	66
	3′-Primer: CTC CTA TGG TAC CTA AAC AT	
TGF-α	5′-Primer: CGC CCT GTT CGC TCT GGG TAT	66
	3′-Primer: AGG AGG TCC GCA TGC TCA CAG	
VEGF	5′-Primer: TCG GGC CTC CGA AAC CAT GA	58
	3′-Primer: CCT GGT GAG AGA TCT GGT TC	
FGF-2	5′-Primer: GGC CAC TTC AAG GAC CCC AAG	58
	3′-Primer: TCA GCT CTT AGC AGA CA	
IGF-1	5′-Primer: ACA TCT CCC ATC TCT CTG GAT TTC CTT TTG	58
	3′-Primer: CCC TCT ACT TGC GTT CTT CAA ATG TAC TTC	

Optimization was performed for each gene-specific primer, confirming that the primers did not produce nonspecific primer-dimmer amplification signal in a control tube lacking template. The primers for 18S rRNA were purchased from a commercial vendor (PE Life Sciences). Quantitative PCR was performed with the ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems) by using 3-stage program parameters provided by the manufacturer as follows: 2 minutes at 50°C, 10 minutes at 95°C, and then 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Liver samples were obtained from at least 4 mice each in EPC-transplanted and control groups, and each of these samples was analyzed in triplicate by quantitative PCR.

Statistical Analysis

Data are expressed as means ± SD. Differences between groups were analyzed by the Mann–Whitney *U* test. Kaplan–Meier analysis with the log-rank test was used for survival comparison between groups. *P* values below .05 were considered indicative of statistical significance.

Results

Morphology of EPC

MNC-derived cells from human peripheral blood that had attached to culture dishes formed multiple cell clusters similar to blood islands and cord-like structures resembling blood vessels on day 7 in culture (Figure 2*A* and *B*). To assess microscopically, May-Giemsa staining was performed on cells removed from dishes at day 14 of culture. Morphology of attaching cells had changed dramatically, showing eccentrically placed nuclei and abundant basophilic cytoplasm (Figure 2*C*). On the other hand, morphology of floating cells had not changed. In addition, mouse MNC attaching to culture dishes showed similar morphology (data not shown).

These attaching cells in human MNC were immunocytochemically positive for Flk-1, Flt-1, Tie-2, CD34 (endothelial cell markers), and CD133 (stem/progenitor cell marker) (Figure 2*D–I*). There were no positive cells in the absence of primary antibodies (data not shown). These results confirmed that we had succeeded in culturing EPC from MNC of human peripheral blood and mouse bone marrow.

Table 3. Real-time-PCR Primer Sequence for Mouse and Human VEGF

Primer	Sequence (5′-3′)
Common	5′-Primer: CCGAAACCATGAACTTTCTGA
Mouse VEGF	3′-Primer: CCATTCATGGGACTTCTGCTC
Human VEGF	3′-Primer: CTTCGTGATGATTCTGCCCTCC

Incorporation of EPC During Liver Regeneration After CCl_4-Induced Liver Injury

Hepatocytes surrounding hepatic central veins showed extensive necrosis after CCl_4 injection (represented by the livers on day 2 in control group; Figure 3*A*). Transplanted human EPC were observed mainly at these necrotic foci near central veins, beginning on day 2 after CCl_4 injection (Figure 3*B*). Some EPC also were observed along hepatic sinusoids beginning on day 2 (Figure 3*C*). In addition, EPC were observed in bone marrow (Figure 3*D*). To clarify how EPC were incorporated into the architecture of hepatic sinusoids, SEC were stained immunohistochemically with CD31 and Flk-1. Human EPC with red fluorescence merged into CD31 green signals (Figure 3*E–G*) or Flk-1 green signals (Figure 3*H–J*), suggesting EPC incorporation into the sinusoidal architecture otherwise made up of SEC.

Hepatocytes proliferated extensively toward hepatic central veins, and no necrotic hepatocytes were observed by day 14 after CCl_4 injection. However, tubular structures, never observed in normal liver, appeared near hepatic central veins. Immunohistochemistry for Tie-2 demonstrated that the tubular structures were formed by cells of endothelial lineage in the livers on day 14 in the

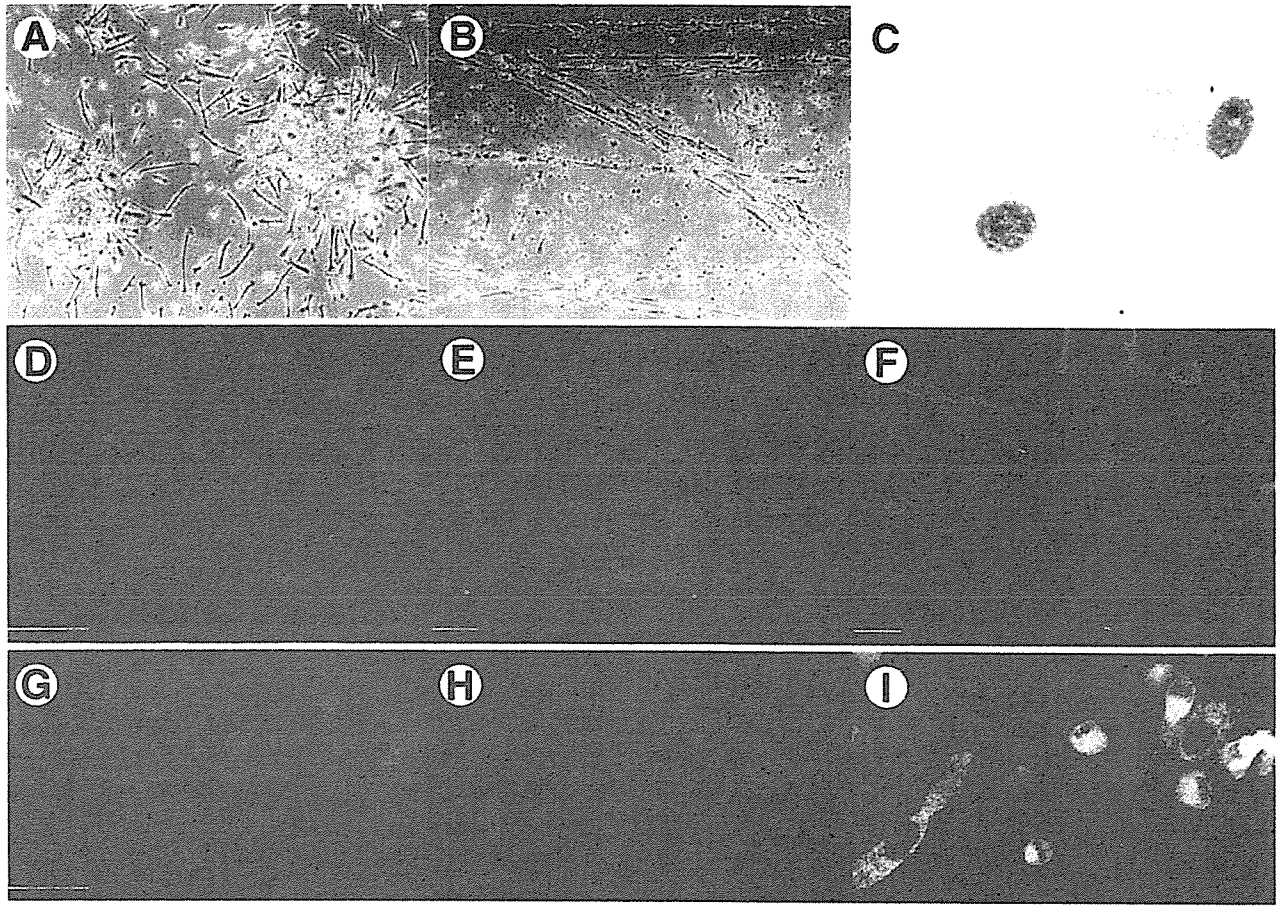

Figure 2. Morphology of human EPC by phase contrast microscopy (*A* and *B*) and by May-Giemsa staining (*C*). Adherent cells were observed to show cluster formation similar to blood islands (*A*) and cord-like structures similar to blood vessels (*B*) during culture, and May-Giemsa staining on day 14 in culture showed the cells to have small, eccentric nuclei and abundant basophilic cytoplasm, which are not shown in human bone marrow or peripheral blood (*C*). Adherent cells expressed Flk-1, Flt-1, and Tie-2 (endothelial cell markers) (*D–F*) and also coexpressed another endothelial cell marker (CD34) and stem/progenitor cell marker (CD133) (*G–I*) on day 7 in culture, showing the characters of EPC. Original magnification in *A*, 200×; *B*, 100×; and C, 400×. *Scale bar* = 10 μm.

human EPC-transplanted group (Figure 3*K*), indicating that the structures could be blood vessels. Moreover, human EPC with red fluorescence formed a portion of these vessel-like structures (Figure 3*L*). Such structures were apparent until day 56 after CCl_4 injection (data not shown).

To elucidate how EPC were attracted to tissues surrounding hepatic central veins, expression of VEGF, a known mobilizing factor for EPC, was studied in regenerating liver. VEGF was detected immunohistochemically in some hepatocytes surrounding central veins on day 2 (represented in the human EPC-transplanted group; Figure 3*M*).

There were no signals in the absence of primary antibodies. In addition, no EPC were observed in intact livers or in other organs (lung and kidney) in mice with liver injury (data not shown).

Improvement of Mouse Survival by EPC Transplantation Following Liver Injury

To clarify the role of EPC in liver regeneration, survival rate was calculated. At first, we compared the rate between human EPC-transplanted and control groups. In the control group, 9 of 14 mice died by day 3 after CCl_4 injection. Only 4 of 14 (28.6%) remained alive on day 7 after CCl_4 injection. However, in the human EPC-transplanted group, 12 of 14 mice (85.7%) remained alive on day 7; only 2 mice had died by day 3. We similarly examined the role of mouse EPC in liver regeneration. In the control group, only 5 of 15 mice (33.3%) remained alive on day 7; however, 12 of 15 mice (80.0%) remained alive in the mouse EPC-transplanted group. Thus, both human and mouse EPC transplantation significantly improved survival at 7 days after CCl_4-induced liver injury (Figure 4*A* and *B*).

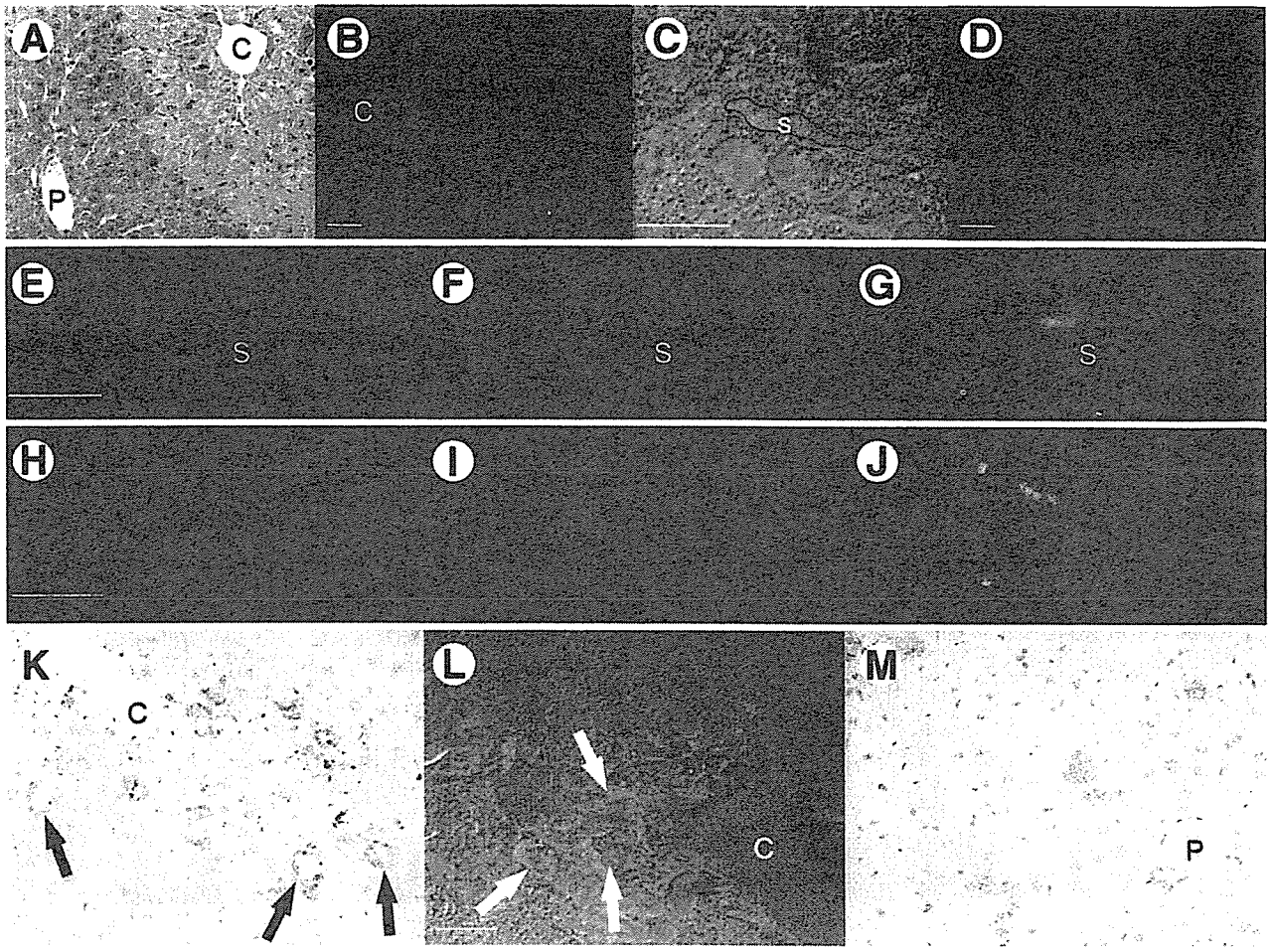

Figure 3. Incorporation of human EPC during liver regeneration after CCl_4-induced liver injury. Hepatocytes surrounding central veins showed extensive necrosis (represented by the livers day 2 in control group, *A*). Most transplanted EPC were observed in this area at day 2 after CCl_4 injection (*B*). Some EPC, tagged by red fluorescence, also were observed along hepatic sinusoids by differential interference microscopy (*C*). In addition, EPC were observed in the bone marrow (*D*). EPC showing red fluorescence were seen to merge into CD31 (*E*, *F*, and *G*) or Flk-1 (*H*, *I*, and *J*) green signals by confocal laser scanning microscopy, suggesting EPC incorporation into the SEC network. Blood vessel-like structures positive for Tie-2 (*K*) were observed surrounding hepatic central veins; at day 14 after CCl_4 injection, these included EPC (*L*). Hepatocytes surrounding central veins expressed VEGF, which is known to mobilize EPC from the bone marrow, at day 2 after CCl_4 injection (*M*). The *open circle* in *C* indicates the hepatic sinusoid area. *Arrows* indicate blood vessel-like structures. Original magnification in *A*, *G*, and *I*, 200×. *Scale bar* = 20 μm. C, central vein; P, portal vein; S, hepatic sinusoid.

Hepatocytic Alteration in Proliferation or Necrosis

We presumed that EPC transplantation could promote proliferation of hepatocytes by day 3 after CCl_4 injection. We therefore compared pathologic findings in livers on day 2 after CCl_4 injection between the control and human EPC-transplanted groups. PCNA staining demonstrated much more active proliferation of hepatocytes in the human EPC-transplanted group than in the control group (Figure 5*A* and *B*). The PCNA-labeling index in the human EPC-transplanted group was significantly greater than in the control group (Figure 5*C*). There were no positive cells in the absence of primary antibodies (data not shown).

On the other hand, necrotic change of the liver on day 2 in the human EPC-transplanted group seemed to be similar to that in the control group (data not shown). Also, there was no significant difference of alanine aminotransferase concentrations on day 2 between the control and human EPC-transplanted groups (n = 10; 10,172 ± 10,262 and 12,548 ± 15,290 U/L, respectively).

Expression of Growth Factors in EPC and Regenerating Liver

Because proliferation of hepatocytes was greatly accelerated, we examined several growth factors that promote liver regeneration. RT-PCR analysis and im-

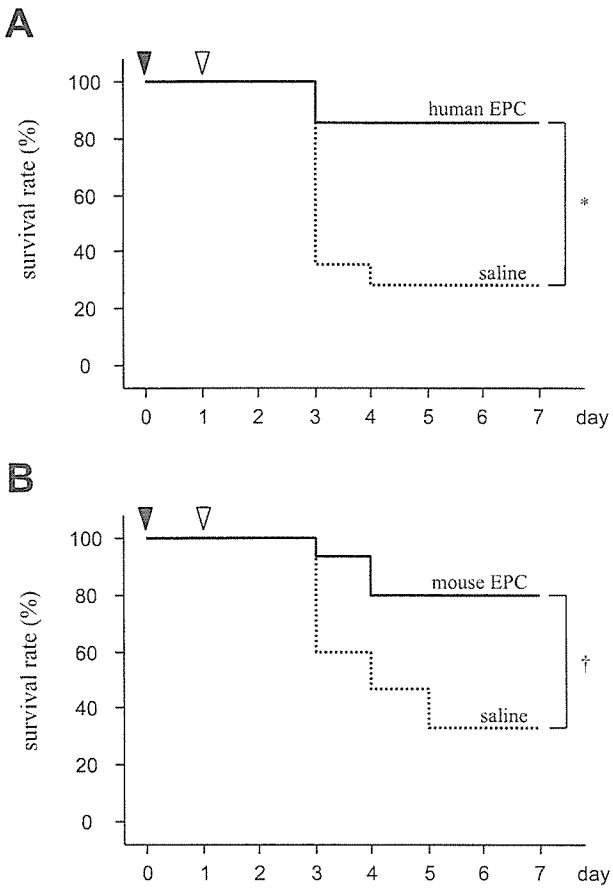

Figure 4. Survival curves after CCl_4 injection in human or mouse EPC-transplanted and control groups. The number of mice alive until day 7 after CCl_4 injection in the human EPC-transplanted group was 12 of 14 (85.7%), whereas that in control group was only 4 of 14 (28.6%). Similarly, the number of mice alive until day 7 in the mouse EPC-transplanted group was 12 of 15 (80.0%), whereas that in the control group was only 5 of 15 (33.3%). Both human and mouse EPC transplantation significantly improved survival after liver injury (*$P <$ .005 and †$P <$ .001, respectively).

munocytochemistry demonstrated messenger RNA (mRNA) (Figure 6*A* and *B*) and proteins (Figure 7*A* to *D*) representing HB-EGF, TGF-α, HGF, VEGF, fibroblast growth factor (FGF)-2, and insulin-like growth factor (IGF)-I in human EPC on day 7 in vitro.

RT-PCR analysis further demonstrated that only in the human EPC-transplanted group, human VEGF mRNA was expressed in regenerating liver on day 2 after CCl_4 injection. In contrast, mouse VEGF mRNA was expressed in regenerating liver in both the human EPC-transplanted and control groups (Figure 8*A*). Furthermore, real-time PCR demonstrated that the amount of mouse VEGF mRNA in the human EPC-transplanted group was significantly greater than in the control group (Figure 8*B*). Similarly, ELISA demonstrated significantly more abundant mouse HGF in regenerating liver at day 2 after CCl_4 injection in the human EPC-transplanted group than in controls (Figure 9).

Discussion

In the present study, we demonstrated that transplanted EPC contributed to liver regeneration in mice by participation in neovascularization and expression of multiple growth factors. In consequence, EPC transplantation significantly accelerated liver regeneration with enhanced proliferative activity of hepatocytes, resulting in the improved survival after chemically induced liver injury.

Reconstruction of hepatic sinusoids as well as hepatocyte proliferation is necessary in liver regeneration. Restoration of sinusoids has been investigated mainly in terms of SEC, the resident endothelial cells. However, because EPC take part in neovascularization in myocardial and hindlimb ischemia,[4–7] and bone marrow-derived cells of presumed endothelial and monocyte lineage are involved in forming sinusoids in regenerating liver after partial hepatectomy,[15] EPC were considered likely to be involved in sinusoid reconstruction. To obtain direct evidence of vasculogenesis by EPC in regenerating liver, we injected EPC obtained from human peripheral blood into spleens of immune-deficient mice. Although most of these transplanted EPC were found in tissues surrounding central veins, some were incorporated into hepatic sinusoids. Subsequently, EPC located in tissues surrounding central veins formed blood vessel–like structures as hepatocytic regeneration progressed. These observations directly documented that EPC contributed to neovascularization in the regenerating liver.

Healing of injured tissue including liver requires neovascularization, and thus acceleration of neovascularization facilitates tissue repair.[18–20] We similarly hypothesized that enhanced neovascularization by EPC transplantation could promote regeneration of the liver, and we demonstrated direct neovascularization by EPC. However, an issue remained concerning whether EPC transplantation would accelerate liver regeneration. In the early stage of liver regeneration, unlike later stages, EPC rarely reconstructed hepatic sinusoids, and essentially all EPC located in necrotic foci surrounding central veins were not forming blood vessels. Nevertheless, hepatocyte proliferation was markedly accelerated by EPC transplantation, even at the early stage. Differences in hepatocyte necrosis and apoptosis between the EPC-transplanted and control group were unlikely; alanine aminotransferase concentrations in peripheral blood were similar between mice

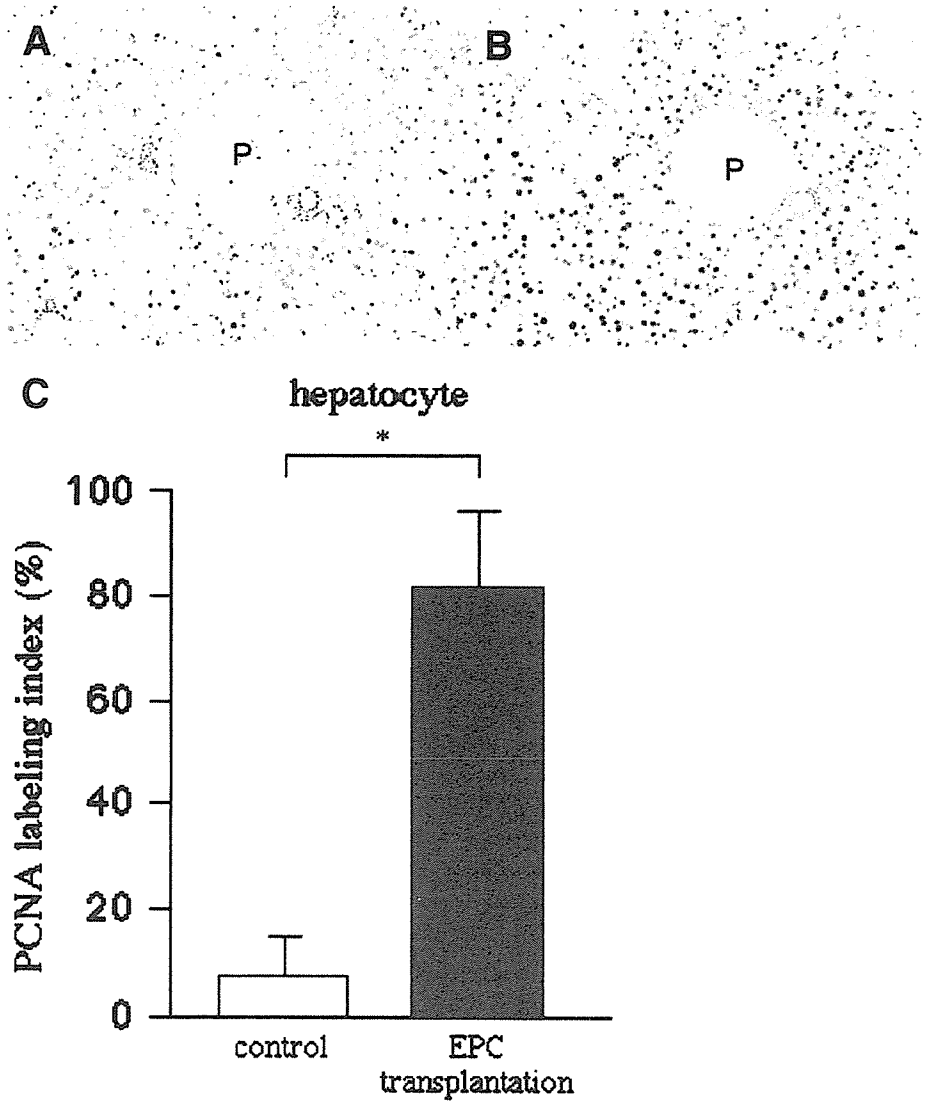

Figure 5. Proliferative activity of hepatocytes in human EPC-transplanted and control groups. PCNA staining demonstrated active proliferation of hepatocytes in the EPC-transplanted group (*A*) compared with much less active hepatocyte proliferation in the control group (*B*). The PCNA-labeling index in hepatocytes in EPC-transplanted mice was significantly greater than in the control group (*$P < .0001$, *C*). Hepatocytes were counted in each of 4 fields from 6 independent experiments in both groups. Original magnification in *A* and *B*, 100×.

receiving and not receiving EPC, whereas no apoptotic hepatocytes were detected by terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate-biotin nick-end labeling in either group (data not shown). We therefore focused on several growth factors known to promote liver regeneration, representing HGF,[21–25] HB-EGF,[24,25] TGF-α,[26,27] and VEGF.[10,11] EPC in culture expressed HGF, HB-EGF, TGF-α, and VEGF and confirmed that transplanted EPC were capable of expressing VEGF mRNA in regenerating liver. Moreover, EPC are likely to have the potential to express a variety of growth factors because EPC are CD34-positive cells, a category that generally has been demonstrated to produce several cytokines and growth factors.[28] In addition, EPC transplantation was shown to induce multiple endogenous growth factors. Mouse HGF protein and VEGF mRNA were elevated in regenerating liver after EPC transplantation. Taken together, we presumed that accelerated hepatic regeneration in the early stage involved growth factors produced by transplanted EPC as well as mouse liver and, later, additionally involved direct neovascularization by transplanted EPC. Moreover, important findings by Matsumoto et al emphasized that liver organogenesis in fetal life was promoted by endothelial cells before they started to function as vessels, and this process was independent of HGF.[29] A similar phenomenon might contribute to postnatal liver regeneration after EPC transplantation.

VEGF has been considered essential for SEC proliferation in liver regeneration.[10,11,13,20,30] Additionally, VEGF recently has been found to be one of the important factors mobilizing EPC from the bone marrow as well as inducing EPC differentiation.[31–36] In our model, hepatocytes in the area surrounding central veins—where

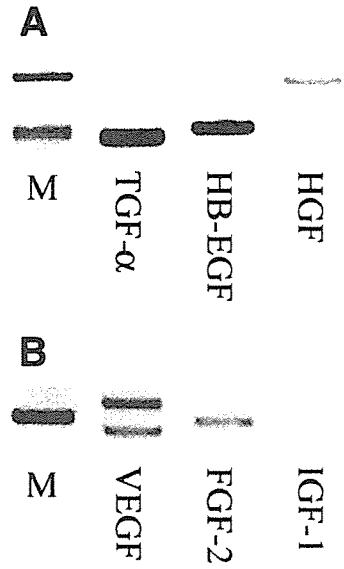

Figure 6. RT-PCR analysis for growth factors in human EPC in vitro. EPC expressed mRNA encoding several growth factors such as HGF, HB-EGF, TGF-α, VEGF, FGF-2, and IGF-1 (*A* and *B*). M, molecular size marker.

transplanted EPC were most evident—expressed VEGF. We therefore believe that, in regenerating liver, VEGF expression by hepatocytes and partly by EPC could regulate not only SEC proliferation but also EPC mobilization and differentiation. Thus, VEGF may be the principal growth factor directing neovascularization (both angiogenesis and vasculogenesis) in liver regeneration. Although how mononuclear cells differentiate into EPC remains unclear, our results suggest one possibility. Whereas EPC from peripheral blood morphologically resembled other mononuclear cells, their appearance by

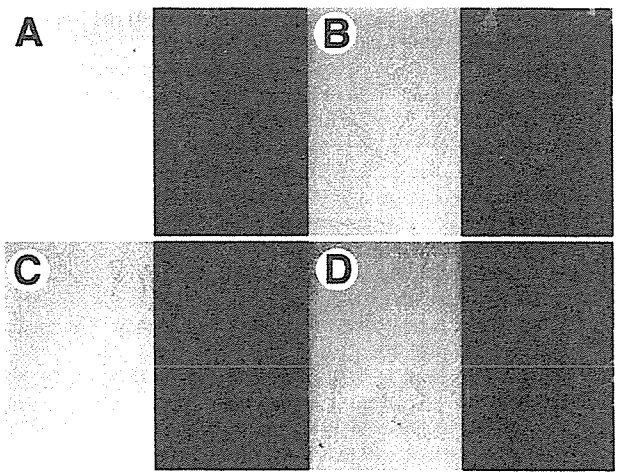

Figure 7. Immunocytochemistry for growth factors performed in human EPC in vitro. EPC expressed several growth factors such as HGF (*A*), TGF-α (*B*) HB-EGF (*C*), and VEGF (*D*). *Scale bar* = 20 μm.

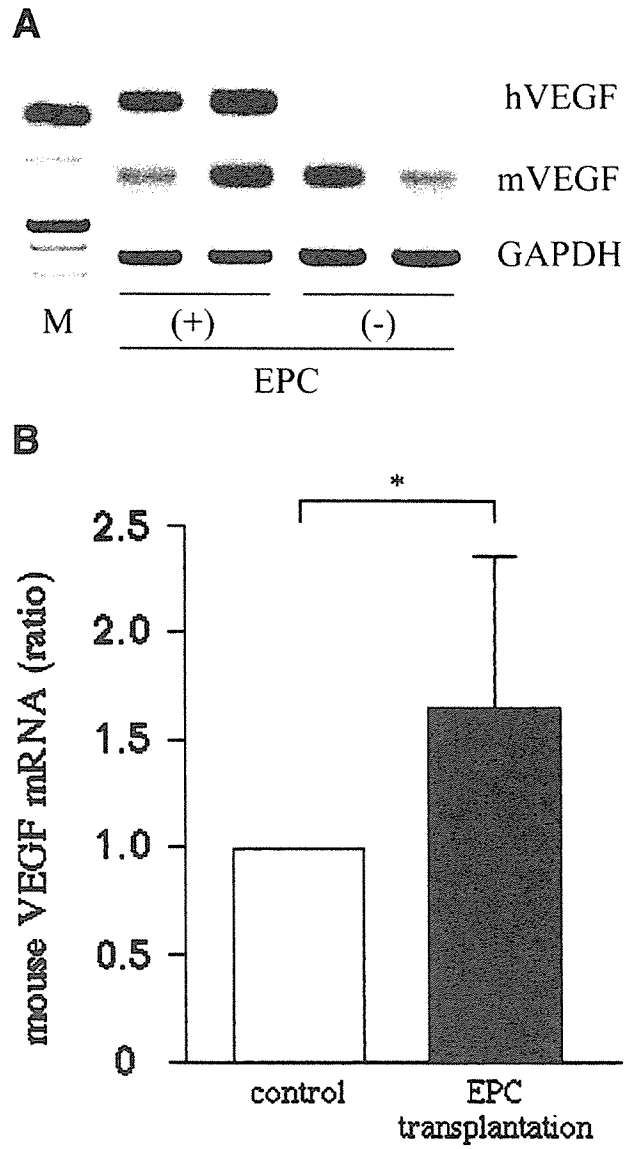

Figure 8. Human and mouse VEGF mRNA analysis by RT-PCR and real-time PCR. RT-PCR detected human VEGF mRNA in livers from the human EPC-transplanted group but not in control-group livers, whereas mouse VEGF mRNA was detectable in livers from both groups (*A*). Real-time quantitative PCR analysis showed that the amount of mouse VEGF mRNA in regenerating liver after EPC transplantation was greater than that in control mice without transplantation (*$P < .005$, *B*). Liver samples were obtained from at least 4 mice in the EPC-transplanted group or control group, and each sample was examined in triplicate by quantitative PCR. M, molecular size marker; hVEGF, human VEGF mRNA; mVEGF, mouse VEGF mRNA.

May-Giemsa staining changed dramatically after EPC attached to surfaces, presumably including extracellular matrix and blood vessel walls. Such EPC morphology was never observed in human bone marrow or peripheral blood.

Although our animal study suggests the possibility of an attractive therapeutic strategy for promoting liver

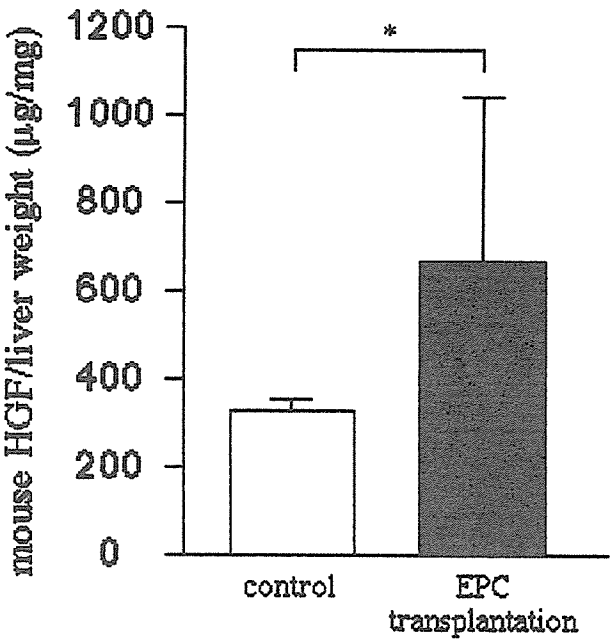

Figure 9. Mouse HGF concentration by ELISA. The amount of mouse HGF in livers from the EPC-transplanted group was greater than that in livers from the control group ($*P < .005$). Liver samples were obtained from 6 mice each in the EPC-transplanted and control groups.

regeneration by EPC transplantation, the very limited size of the EPC population represents an important obstacle to clinical application. EPC transplantation therefore should require a greater number of EPC from other resources such as bone marrow,[8] cord blood,[5] or embryonic stem cells,[37,38] whereas EPC efficacy might be enhanced by combination with drugs and gene transfer. EPC preconditioned with hypoxia[39] or transduced by VEGF[36] or telomerase reverse-transcriptase gene[40] recently have shown more active proliferation and migration, and more resistance to apoptosis, than ordinary EPC, improving salvage of ischemic hindlimbs. In addition, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor was found to promote EPC proliferation, migration, and resistance to apoptosis.[41–43] Therefore, EPC transplantation combined with HMG-CoA reductase inhibitor, hypoxic preconditioning of EPC during ex vivo expansion, and gene transfer of VEGF and telomerase reverse transcriptase to EPC might result in an approach showing promise for clinical application.

In conclusion, this was the first demonstration that growth factors derived from transplanted EPC and induced in mouse liver, as well as vasculogenesis involving transplanted EPC, significantly accelerated liver regeneration and ultimately improved survival following chemically induced liver injury. These results suggested that EPC transplantation would become a new therapeutic strategy for promoting liver regeneration after fulminant and severe hepatitis or after surgical hepatectomy. However, major problems first must be overcome, particularly the present need for large numbers of cells.

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Surveillance Program for Early Detection of Hepatocellular Carcinoma in Japan

Results of Specialized Department of Liver Disease

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Objective: Surveillance of cirrhotic patients enables early detection of hepatocellular carcinoma (HCC) and possibly prolongs survival. The aim of this study was to explore whether early-stage HCC can be detected earlier at a specialized department of liver disease than in other institutions.

Methods: The study subjects were 574 patients with HCC. Patients were subdivided into 3 groups according to the manner of HCC detection: group A, HCC was detected in 91 patients during periodic examination at Kurume University School of Medicine; group B, HCC was detected in 301 patients during periodic examination at other institutions; group C, HCC was detected incidentally or because of symptoms in 182 patients.

Results: The HCC detected in group A was significantly of smaller size (20.4 mm) compared with groups B (27.1 mm, $P < 0.0001$) and C (57.8 mm, $P < 0.0001$). The frequency of receiving treatment (surgery or local ablation therapy) was significantly higher in group A (73%) than in groups B (52%, $P = 0.002$) and C (26%, $P < 0.0001$). The 5-year survival rates were 52% for group A, 40% for group B, and 23% for group C, respectively. The survival of group A was significantly better than that of groups B ($P = 0.0157$) and C ($P < 0.0001$).

Conclusions: Surveillance for HCC at specialized Department of Liver Disease can detect early-stage HCC, resulting in a higher chance of receiving promising treatment.

Key Words: hepatocellular carcinoma, surveillance, ultrasonography, computed tomography, tumor markers

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Hepatocellular carcinoma (HCC) is the fifth most common neoplasm in the world, and the third most common cause of cancer-related death.[1] HCC has become the leading cause of death among patients with liver cirrhosis.[2] The incidence of HCC has increased in the United States over the past 2 decades.[3] HCC commonly occurs in patients with chronic liver diseases related to hepatitis C virus (HCV) or hepatitis B virus (HBV) and the incidence of HCC in patients with HCV was reported to be 1.5% to 8% per annum.[4–7] Several studies have shown that surveillance with ultrasonography (US) and α-fetoprotein (AFP) for patients with liver cirrhosis can detect early-stage HCC, resulting in higher chance of receiving early treatment.[4–11] However, some studies showed that surveillance for HCC has a limited value in prolonging survival of patients with HCC including cost effectiveness,[12,13] and there is no randomized controlled trial to establish the value of surveillance of HCC in increasing survival of patients with chronic liver disease. Nowadays, such a study is almost impossible for ethical reasons. Discrepancies in the results of surveillance of HCC are related to differences in the incidence of HCC, target population of surveillance, frequency of surveillance, effective treatment of HCC, management of liver cirrhosis, and possibly also US equipment and skill of US examiner. The improvement of US equipment and increased proficiency of US examiner for surveillance of HCC have allowed early diagnosis of HCC, and resulted in prolonging survival of patients with liver cirrhosis over 3 quinquennia.[10] US equipment and skill of US examiner vary among institutions. However, no controlled trial has compared the results of surveillance of HCC among institutions.

Three-phase computed tomography (CT) and magnetic resonance imaging (MRI) might be potentially more sensitive and specific for the diagnosis of HCC.[14] Studies from the United States indicated that the screening for HCC with CT could be a cost effective strategy in transplant-eligible patients with cirrhosis.[15] Des-γ-carboxy prothrombin (DCP), a new tumor marker of HCC is more specific or equally specific to AFP.[16,17] However, surveillance studies using 3-phase CT, MRI, and DCP have not been reported.

The aims of the present study were (1) to determine differences in detecting early-stage HCC among various departments of liver disease, (2) if such differences have an impact on survival of patients with chronic liver disease, and (3) compare the values of regular 3-phase CT or regular DCP and conventional method of surveillance program of HCC in the detection of early-stage HCC.

PATIENTS AND METHODS

Patients

The study subjects were 574 Japanese patients with HCC diagnosed at Kurume University School of Medicine between January 1995 and December 2000. The diagnosis of HCC was established by histopathology and/or imaging studies (US, CT, angiography, CT-angiography, and MRI), and/or based on high plasma levels of tumor markers such as AFP and DCP. Patients were subdivided into 3 groups according to the manner of HCC discovery: group A, 91 patients were found to have HCC during periodic follow-up examination at Kurume University School of Medicine; group B, 301 patients were found to have HCC during periodic follow-up examination in other institutions; and group C, 182 patients were found to have HCC incidentally or because of symptoms.

Surveillance Program

Surveillance of 91 patients of group A included patients with chronic liver disease irrespective of age, liver cirrhosis, or etiology (HCV, HBV, alcoholic and other chronic liver diseases). Regular surveillance program of 91 patients of group A was as follows: US + AFP, 20 patients; US + AFP + DCP, 20 patients; US + AFP + CT, 15 patients; and US + AFP + CT + DCP, 36 patients. The frequency of monitoring using US, AFP, CT, DCP were 3, 6 to 12, and 3 to 6 months, respectively. During the subsequent surveillance period, imaging studies and tumor markers, together with physical examination and routine biochemical test, were repeated every 3 months. If 1 diagnostic modality indicated possible HCC, the other modalities were then performed on an out-patient basis. When nodular liver lesion was depicted by US or CT in such patients, they were admitted to Kurume University School of Medicine, and the diagnosis of HCC was confirmed by histopathology and/or imaging studies conducted based on high plasma levels of tumor markers.

The 301 patients of group B were found to have nodular liver lesions during periodic follow-up examination at other institutions at least 6-month interval by means of direct interview of the patients. The surveillance program of the 301 patients of group B was unknown. Classification of 182 patients as group C was based on finding a nodular liver lesion incidentally or at examination for symptoms and interview of patients but not at periodic follow-up examination.

Treatment Strategy

When a diagnosis of HCC was established at Kurume University School of Medicine, the following treatment options were assessed. Liver transplantation (LT)[18,19] was not considered because of very small number of donor resources and insurance system in Japan from January 1995 to December 2000. (1) Hepatic resection (HR)[20] was assessed especially for patients with localized HCC and preserved hepatic reserve capacity. (2) Nonsurgical treatments, such as percutaneous ethanol injection (PEI),[21] microwave coagulation therapy (MCT),[22] radiofrequency ablation (RFA),[23,24] transarterial chemoembolization (TACE),[25] hepatic arterial infusion chemotherapy (HAIC),[26] and systemic chemotherapy[27] were assessed when HR was contraindicated or the patient refused surgical treatment. The most appropriate therapeutic procedure was selected according to the tumor status and the underlying liver cirrhosis. Local ablation therapies (LAT) such as, PEI, MCT, and RFA were considered in patients with 1-3 tumor nodules, each measuring $\leq$30 mm in diameter that were devoid of vascular invasion and not associated with extrahepatic metastasis. (3) TACE, HAIC, or systemic chemotherapy was considered in patients with maximum tumor size of $>$ 30 mm, number of tumors $>$ 3, presence of vascular invasion and/or presence of extrahepatic metastasis. (4) Best supportive care was assessed when patient had little hepatic reserve capacity or patient refused any treatment of HCC.

Outcome Measures

Outcome measures were analyzed retrospectively in groups A to C as follows: (1) tumor characteristics including size and number of HCC nodules, presence of vascular invasion, and presence of extrahepatic metastasis; (2) UNOS (The United Network for Organ Sharing) criteria for HCC[28]; (3) treatment of HCC; and (4) cumulative survival of patients with HCC.

Differences of Surveillance Program at Kurume University School of Medicine

In 91 patients of group A, 51 patients underwent regular CT (15 US + AFP + CT and 36 US + AFP + CT + DCP) and 56 patients underwent regular DCP (20 US + AFP + DCP and 36 US + AFP + CT + DCP) in addition to US and AFP for surveillance program of HCC, respectively. (1) Tumor characteristics; (2) UNOS criteria for HCC; (3) treatment of HCC; and (4) cumulative survival of patients with HCC was also compared in 51 patients with regular CT [regular CT (+) group] and 40 patients without regular CT [regular CT (−) group], and in 56 patients with regular DCP [regular DCP (+) group] and 36 patients without regular DCP [regular DCP (−) group].

Statistical Analysis

We used the χ^2, Fisher exact, and Mann-Whitney tests, where appropriate, to evaluate differences in clinical features of patients and in tumor characteristics. Survival was analyzed by the Kaplan-Meier method[29] and survival curves were compared by the log-rank test. Survival was

TABLE 1. Clinical Profile of 572 Patients With HCC

	Group A	Group B	Group C
No. patients	91	301	182
Age (y, mean ± SD)	65.4 ± 7.4	65.2 ± 8.8	63.5 ± 9.3
Sex			
Male (%)	54 (59)	214 (71)	158 (87)
Female (%)	37 (41)	87 (29)	24 (13)
		$P = 0.035$	$P < 0.0001$
			$P < 0.0001$*
Etiology			
HCV-positive (%)	82 (90)	258 (86)	135 (74)
HBV-positive (%)	4 (4)	30 (10)	31 (17)
HCV-negative and HBV-negative (%)	5 (6)	13 (4)	16 (9)
			$P = 0.006$
			$P = 0.006$*
Total bilirubin (mg/dL: mean ± SD)	1.27 ± 0.64	1.22 ± 0.78	1.12 ± 0.86
Albumin (g/dL: mean ± SD)	3.39 ± 0.49	3.45 ± 0.47	3.50 ± 0.45
Child pugh class			
A (%)	53 (58)	186 (62)	126 (69)
B or C (%)	38 (42)	115 (38)	56 (31)
AFP (ng/mL)			
0 to 100 (%)	68 (75)	181 (60)	91 (50)
> 100 (%)	23 (25)	120 (40)	91 (50)
		$P = 0.011$	$P = 0.030$
			$P < 0.0001$*
DCP (mAU/mL)			
0 to 40 (%)	70 (77)	188 (62)	55 (30)
> 40 (%)	21 (23)	113 (38)	127 (70)

*Group B versus group C.

confirmed up to September 30, 2004. The statistical software package SPSS for Windows (version 10.0, SPSS Inc, Chicago, IL) was used for data analysis. A P value of < 0.05 was considered significant.

RESULTS

Patient Characteristics

Table 1 summarizes the clinical profile of 574 patients with HCC. The 3 groups were comparable for age, serum levels of total bilirubin and albumin, and Child Pugh class, whereas they significantly differed for sex (group A vs. B: $P = 0.035$; group A vs. C: $P < 0.0001$; group B vs. C: $P < 0.0001$) and etiology of liver disease (group A vs. C: $P = 0.006$; group B vs. C: $P = 0.006$). Serum levels of AFP (> 100 ng/mL) and DCP (> 40 mAU/mL) were significantly higher in group C than in groups A and B, and significantly higher in group B than in group A (AFP; group A vs. group B: $P = 0.011$, group A vs. group C: $P < 0.0001$, group B vs. group C: $P = 0.030$. DCP; group A vs. group B: $P = 0.011$, group A vs. group C: $P < 0.0001$, group B vs. group C: $P < 0.0001$).

HCC Features

The characteristics of HCC in the three groups are listed in Table 2. Significantly smaller size and fewer HCC nodules were detected in group A than in groups B and C, and significantly smaller in group B than in group C (tumor size: A, B, C; 20.4, 27.1, 57.8 mm, respectively, group A vs. group B: $P < 0.0001$; group A vs. group C: $P < 0.0001$; group B vs. group C: $P < 0.0001$. Number of tumors; group A vs. group B: $P < 0.0001$; group A vs. group C: $P < 0.0001$; group B vs. group C: $P < 0.0001$). A significantly higher proportion of tumors showed vascular invasion in group C than in groups A and B, and significantly higher in group B than in group A (group A vs. group B: $P = 0.020$; group A vs. group C: $P < 0.0001$; group B vs. group C: $P < 0.0001$). Extrahepatic metastasis was noted in 9 patients. A significantly higher proportion of extrahepatic metastasis was noted in group C than in groups A and B (group A vs. group C: $P = 0.042$; group B vs. group C: $P = 0.001$).

UNOS Criteria and Treatment

Of the 574, 334 patients (58%) presented with HCC within UNOS T2 criteria (Table 2). A significantly higher proportion of patients presented with HCC within UNOS T2 criteria in group A (91%) compared with group B (68%) and group C (26%), and in group B compared with group C (group A vs. group B: $P < 0.0001$; group A vs. group C: $P < 0.0001$; group B vs. group C: $P < 0.0001$). With regard to treatment, 10 (11%), 20 (7%), and 16 (9%) of groups A, B, and C were treated with HR, respectively. Furthermore, 56 (62%), 137 (45%), and 31 (17%) of group A, B, and C were treated with LAT including PEI, MCT, and RFA, respectively. In addition, 21 (23%), 132 (44%), and 122 (67%) of groups A, B, and C were treated with interventional radiology including TACE and HAIC, respectively. For other therapies, 2 of group C were treated with systemic chemotherapy, and 4 (4%), 12 (4%), and 13 (7%) of groups A, B, and C were