Branched Chain Amino Acids Enhance the Maturation and Function of Myeloid Dendritic Cells *Ex Vivo* in Patients with Advanced Cirrhosis

Eiji Kakazu,[1] Yoshiyuki Ueno,[1] Yasuteru Kondo,[1] Koji Fukushima,[1] Masaaki Shiina,[1] Jun Inoue,[1] Keiichi Tamai,[1] Masashi Ninomiya,[1] and Tooru Shimosegawa[1]

An imbalance of plasma amino acids is observed in patients with advanced cirrhosis. The aim of this study was to investigate the influence of the extracellular amino acid imbalance on the function of myeloid dendritic cells (DCs) in patients with advanced cirrhosis. We made a serum-free culture medium consistent with the average concentration of plasma amino acids from healthy controls (HC, n = 25) or patients with advanced cirrhosis (LC, n = 43) to reflect more closely the actual environment of the living body. We compared the phenotypical and biological functions of blood dendritic cells antigen-positive dendritic cells (BDCA+ DCs) and monocyte-derived dendritic cells (MoDCs) from LC and HC with these media. After adding stimulants, the CD83 and CD86 expressions of DCs from LC were lower than those from HC. In both HC and LC, both CD83 and CD86 expressions of DCs stimulated under the cirrhotic medium were lower than under the control medium. This phenomenon was accompanied by a suppression of the mammalian target of rapamycin (mTOR)/S6K-signaling pathways. The interleukin 12 (IL-12) production in the cirrhotic medium was significantly lower than in the control medium and increased when valine or leucine was added to the medium. In patients with advanced cirrhosis, peripheral blood mononuclear cells stimulated in the autologous plasma after oral administration of branched-chain amino acid (BCAA) granules had significantly increased interferon gamma production. *Conclusion:* In advanced cirrhosis, there is impairment of the function and maturation of DCs, which has been shown to be related to an imbalance in the extracellular amino acid profile. Elevating the extracellular concentration of BCAAs *ex vivo* in patients with advanced cirrhosis improved the function of DCs. (HEPATOLOGY 2009;50:1936-1945.)

Cirrhosis makes it increasingly difficult for the liver to carry out its essential functions, such as detoxifying harmful substances and manufacturing vital nutrients. Cirrhosis progresses to decompensated cirrhosis and ultimately liver failure because of a lack of suitable treatment. Not only hepatocellular carcinoma but also nosocomical infections, such as spontaneous bacterial peritonitis (SBP) or pneumonia, are frequent clinical complications in these immune-compromised patients.[1] In patients with advanced cirrhosis, various metabolic disorders involving glucose, protein-amino acids, lipids, vitamins, and minerals might appear. Furthermore, an imbalance of plasma amino acids, with decreased levels of branched-chain amino acids (BCAAs)

Abbreviations: AAA, aromatic amino acid; ACM, advanced cirrhotic media; APC, antigen-presenting cell; BCAA, branched-chain amino acid; BDCA, blood dendritic cells antigen; DC, dendritic cell; HCM, healthy control media; IFN-γ, interferon gamma; IL, interleukin; MLR, mixed lymphocytes reaction; MoDC, monocyte-derived dendritic cell; mTOR, mammalian target of rapamycin; NKT, natural killer T; PBMC, peripheral blood mononuclear cell; SBP, spontaneous bacterial peritonitis.

From the Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan.

Received February 19, 2009; accepted August 11, 2009.

Supported in part by a grant-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to E.K.) (21790643), and by Health and Labour Sciences Research Grants for the Research on Measures for Intractable Diseases (from the Ministry of Health, Labour and Welfare of Japan; to Y.U.).

Address reprint requests to: Yoshiyuki Ueno, Division of Gastroenterology, Tohoku University Graduate School of Medicine, 1-1 Seiryo, Aobaku, Sendai, 980-8574, Japan. E-mail: yueno@mail.tains.tohoku.ac.jp; fax: 81-22-717-7177.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.23248

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

and increased levels of aromatic amino acids (AAAs), is commonly seen in patients with advanced cirrhosis.[2] In clinical situations, long-term nutritional supplementation with oral BCAA has been shown to be useful to prevent progressive hepatic failure and to improve surrogate markers and the perceived health status.[3,4] Moreover, the oral administration of BCAA granules was reported to inhibit hepatic carcinogenesis in patients with compensated cirrhosis.[5,6]

On the one hand, it has become clear that amino acids are not only important as substrates for various metabolic pathways but also activate a nutrient-sensitive signaling pathway in synergy with insulin.[7-10] The mammalian target of rapamycin (mTOR) signaling pathway is one of the most representative pathways, and this pathway has been shown to act as a major effector of cell growth and proliferation by way of the regulation of protein synthesis.[7-9] The phosphorylation of downstream effectors of mTOR is inhibited by rapamycin and activated by BCAA, especially by leucine,[11-13] although little is known about the impact of changes in the extracellular amino acid levels on the immune system.[14] Recently, we have shown that extracellular BCAAs, especially valine, regulate the maturation and function of monocyte-derived dendritic cells (MoDCs).[15] Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that stimulate innate and adaptive immune reactions by priming other types of blood cells. Typically, immature DCs migrate to lymphoid tissues and present antigenic peptides to naive T cells.[16] The mature DCs, which characteristically express CD83,[17] can rapidly activate other innate immune cells including natural killer (NK) cells and natural killer T (NKT) cells through the production of immunomodulatory cytokines such as interleukin (IL)-10 and IL-12. Several studies have reported that the immunological abnormalities occurring in cirrhosis,[18,19] such as a depressed reticuloendothelial system, neutrophil dysfunction, reduced serum complement, and low bactericidal function, account for the increased susceptibility of patients with cirrhosis to bacterial seeding and diffusion, and for the impaired functions of DCs in patients with liver cirrhosis.[15,20,21] However, it is not clear why the responses of immune cells, particularly DCs, are suppressed in patients with cirrhosis.

Roswell Park Memorial Institute medium 1640 (RPMI 1640) with human or bovine serum is typically used to culture peripheral blood mononuclear cells (PBMCs) or DCs and examine the function. The concentrations of almost all the amino acids in RPMI 1640 are higher than those typically found in the plasma of healthy adult humans. Accordingly, there are large differences between the amino acids of living bodies and those of culture systems. The concentration of amino acids except BCAAs in the medium used in our previous study was higher than that of plasma *in vivo*.[15] Furthermore, various types of amino acid imbalance actually appear in the plasma of patients with advanced cirrhosis. The aim of the study, therefore, was to investigate the influence of the extracellular amino acid imbalance observed in patients with advanced cirrhosis on the function of DCs using a serum-free culture medium consistent with the average concentration of plasma amino acids from healthy volunteers (healthy control media, HCM) or patients with advanced cirrhosis (advanced cirrhotic media, ACM) to reflect more closely the actual environment of the living body. Furthermore, we investigated whether oral administration of BCAA granules could enhance the responses of immune cells in patients with advanced cirrhosis.

Patients and Methods

Serum-Free Culture Media. The concentrations of the plasma amino acids from fasting healthy volunteers (n = 25), chronic hepatitis (n = 14), and patients with cirrhosis (n = 60) were measured by high-performance liquid chromatography (HPLC) in the early morning (Table 1). Briefly, sulfosalicylic acid was added to plasma to a final concentration of 5%. The samples were then placed on ice for 15 minutes followed by centrifugation to remove precipitated proteins. The extracts were then analyzed for the amino acid content with a JLC-500/V (Japan Electron Optics Laboratories, Tokyo, Japan). Also, these patients with cirrhosis were classified according to the Child-Pugh classification. We defined as Child-Pugh grade B or C the patients with advanced cirrhosis (n = 43: hepatitis c virus [HCV] n = 22; primary biliary cirrhosis [PBC] n = 5; alcoholic n = 3; nonalcoholic steatohepatitis [NASH] n = 3; hepatitis b virus [HBV] n = 2; primary sclerosing cholangitis [PSC] n = 2; HCV+HBV n = 1; autoimmune hepatitis [AIH] n = 1; Wilson's disease n = 1; Budd-Chiari syndrome n = 1; cryptogenic n = 2). A serum-free culture medium consistent with the average concentration of plasma amino acids from healthy volunteers was defined as the HCM; whereas that from patients with advanced cirrhosis was defined as the ACM (Table 2). Other components except amino acids were identical among media. We verified that there was no difference between the theoretical value and actual value in HCM and ACM. We cultured PBMCs under the two media with stimulant for 48 hours and measured the amino acid concentrations of these media. There was no difference in the concentrations of amino acids before and after culture in these media. The viability of PBMCs was determined using Annexin V^{FITC}, with dead cells identi-

Table 1. Aminogram for the Plasma in Chronic Hepatitis Patients and Patients with Cirrhosis

	HC (n=25)	CH (n=14)	Child A (n=17)	Child B (n=19)	Child C (n=24)
Glycine	225	250	205	234	313
Alanine	391	400	311	317	339
Serine	119	135	139	137	169
Threonine	142	139	137	135	165
Cystine	38	54	63	62	73
Methionine	29	31	40	60	68
Glutamine	564	585	616	642	739
Asparagine	51	57	62	58	77*
Glutamic acid	42	70	62	65	47
Aspartic acid	3	3	5	4	3
Valine	249	243	222	195†	164†
Leucine	132	141	120	110	93†
Isoleucine	76	71	63	56	51†
Phenylalanine	63	70	80	89	99*
Tyrosine	65	81	111	112	151*
Tryptophan	62	52	52	43	47
Lysine	183	223	219	199	179
Arginine	78	79	94	93	100
Histidine	83	90	77	81	93
Proline	204	163	142	165	202
Fischer's ratio	3.57	3.01	2.36†	1.95†	1.27†

The concentrations of plasma amino acids from fasting healthy volunteers (n=25), chronic hepatitis (n=14) and patients with cirrhosis (n=60) were measured by HPLC in the early morning after fasting. Also, these patients with cirrhosis were classified according to the Child-Pugh classification. Amino acid concentrations are expressed in nmol/mL.

*$P < 0.01$ increased. †$P < 0.01$ decreased. Fischer's ratio means: Valine+Leucine+Isoleucine / Tyrosine+Phenylalanine †decrease *increase $P < 0.01$ vs. CH (the data were analyzed with ANOVA and Dunnett's post-hoc procedure).

fied by propidium iodide (PI) staining (Annexin V-FITC Apoptosis Detection Kit, BioVision, Mountain View, CA), according to the manufacturer's instructions. We confirmed the viability of PBMCs cultured in HCM and ACM equal to that of complete culture medium (CCM) and X-VIVO 10 (Cambrex Bio Science Walkersville, Walkersville, MD). The percentages of living cells were 78.7 ± 0.67, 77.7 ± 2.2, 71.7 ± 0.67, and 74.7 ± 0.33 for HCM, ACM, CCM, and X-VIVO10, respectively. The culture media, CCM, and other depleted media were made as described.[15]

Patients and Healthy Volunteers. We selected 15 patients with cirrhosis for *in vitro* or *ex vivo* studies (Table 3). All of these patients were inpatients. There were no significant differences on clinical and laboratory findings in this population compared to the 43 patients with advanced cirrhosis (Table 1): age 60.4 ± 12.8 versus 59.1 ± 11.3; aspartate aminotransferase (AST) 78.8 ± 45.4 IU/L versus 96.3 ± 65.0 IU/L; alanine aminotransferase (ALT) 47.6 ± 25.2 IU/L versus 54.3 ± 36.7 IU/L; total bilirubin 4.5 ± 5.36 mg/dL versus 3.94 ± 3.70 mg/dL; albumin 2.80 ± 0.51 g/dL versus 2.85 ± 0.55 g/dL; prothombin time / international normalized ratio (PT-INR) 1.54 ± 0.39 versus 1.37 ± 0.29; PLT 93.9 ± 68.7 × $10^3/\mu L$ versus 113.1 ± 54.2 × $10^3/\mu L$; Child Pugh score 9.0 ± 1.77 versus 8.6 ± 2.10; Model for End-Stage Liver Disease (MELD) score 11.9 ± 5.55 versus 11.2 ± 4.23; plasma Fischer's ratio 1.56 ± 0.77 versus 1.65 ± 0.57. The MELD score[22] was calculated by an online worksheet available on the Internet at www.mayoclinic.org/meld/mayomodel5.html. None of the patients had clinical or laboratory findings compatible with bacterial infection when we collected PBMCs from the patients. Written informed consent was obtained from each individual and the study protocol was approved by the Ethics Committee of Tohoku University School of Medicine (2003-326, 2008-337).

BDCA+ DCs Maturation and MoDCs Generation. PBMCs were separated from the peripheral blood of HC and LC by centrifugation on a density gradient. The blood dendritic cells antigen-positive dendritic cells (BDCA+ DCs) and the CD14-positive monocytes were isolated from PBMCs using magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). BDCA1+ DCs were cultured at a density of 2.5 × 10^5 cells/well in 96-well flat-bottom plates (Corning, NY) for 48 hours with 1,000 U/mL GM-CSF (PreproTech, London, UK), 500 U/mL (hu) IL-4 in each media. At 24 hours culture,

Table 2. Serum-Free Culture Media Used in This Study (nmol/mL)

	CCM	HCM	ACM
Glycine	400	225	280
L-Alanine	400	391	307
L-Serine	400	119	151
L-Threonine	800	142	138
L-Cystine 2HCl	200	38	67
L-Methionine	200	29	75
L-Glutamine	4000	564	689
L-Asparagine	400	51	64
L-Glutamic Acid	400	42	53
L-Aspartic Acid	400	3	4
L-Valine	800	249	175
L-Leucine	800	132	100
L-Isoleucine	800	76	53
L-Phenylalanine	400	63	99
L-Tyrosine	400	65	133
L-Tryptophan	80	62	45
L-Lysine-HCl	800	183	184
L-Arginine-HCl	400	78	92
L-Histidine HCl-H2O	200	83	85
L-Proline	400	204	176
Fischer's ratio	3.00	3.57	1.42

Complete culture medium (CCM) contains 20 amino acids that are relevant to the make-up of mammalian proteins. HCM (healthy control medium): consistent with the average concentration of plasma amino acids from healthy volunteers (n=25). ACM (advanced cirrhotic medium): consistent with the average concentration of plasma amino acids from patients with advanced cirrhosis (Child-Pugh grade B or C, n=43). The amino acid concentrations are expressed in nmol/mL. Fischer's ratio means: Valine+Leucine+Isoleucine / Tyrosine+Phenylalanine.

Table 3. Characteristics of Study Participants

Patient Number	Disease	Sex	Age (years)	AST/ALT	Total Bilirubin	Albumin	PT-INR	PLT	Child-Pugh Classification	MELD Score	Plasma Fischer's Ratio	BCAA Medication
1	LC-C	M	71	116/61	0.8	3.3	1.09	149	A	6	2.49	-
2	LC-C+HCC	M	70	73/46	1.5	2.3	1.15	75	B	6	2.26	-
3	LC-C+HCC	F	80	72/55	1.3	2.8	1.19	144	B	9	NA	+
4	LC-C	M	42	52/38	4.2	1.8	1.79	79	C	16	0.99	+
5	LC-C+HCC	F	61	238/98	6.3	2.9	1.65	76	B	18	2.74	+
6	PBC	F	43	241/144	12.3	2.8	1.32	152	C	18	1.57	-
7	LC-C	M	56	71/45	2.2	3.7	1.24	81	B	10	1.90	+
8	LC-C	M	48	111/109	1.6	3.7	1.08	81	A	8	NA	-
9	LC-C	F	60	25/5	11.6	3.2	2.05	83	C	15	0.88	+
10	LC-C+HCC	F	69	68/40	1.3	2.8	1.17	132	B	7	1.81	-
11	non B non C	F	44	28/18	2.4	2.6	1.54	122	C	8	1.31	+
12	PBC	F	62	130/49	6.8	2.0	1.33	120	C	8	1.43	+
13	PBC	F	62	83/30	2.3	2.5	1.11	207	B	13	1.29	+
14	Alcoholic	M	54	53/24	2.5	3.1	1.60	219	C	14	1.24	+
15	LC-C+HCC	M	65	83/53	2.0	3.2	1.29	96	B	12	1.52	+

LC-C, liver cirrhosis due to HCV; HCC, hepatocellular carcinoma; PBC, primary biliary cirrhosis; NASH, nonalcoholic steatohepatitis; NA, not available; PLT, platelet counts ($x10^3/\mu L$); PT-INR, prothrombin time-international normalized ratio; AST/ALT, aspartate aminotransferase / alanine aminotransferase (IU/L); total bilirubin (mg/dL); albumin (g/dL); Fischer's ratio: Valine+Leucine+Isoleucine / Tyrosine+Phenylalanine.

DCs were stimulated by 500 ng/mL lipopolysaccharide (LPS; *Escherichia coli* 026:B6; Sigma, St. Louis, MO) or polyinosinic:polycytidylic acid (polyI:C) (30 μg/mL). Monocytes were cultured at a density of 3.0×10^5 cells/well with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 for 6 days in CCM. On day 6 we changed the medium from CCM to HCM or ACM with poly(I:C) and the culture was continued for an additional 48 hours.

Surface Marker Analysis. DCs were harvested and labeled with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled monoclonal antibodies (mAbs) (antihuman CD14, CD40, CD83, CD86, CD98, HLA-DR, or the relevant isotype controls; BD PharMingen, San Diego, CA) according to the manufacturer's instructions. Using a FACS Calibur (BD Immunocytometry Systems, San Diego, CA) flow cytometer, surface marker expressions were analyzed using the CellQuest (BD Immunocytometry Systems) program.

Phagocytosis Assay with Dextran. To evaluate the endocytosis potential of DCs, 1 mg/mL of FITC-dextran was supplied to 2.5×10^5 DCs that were then incubated for 30 minutes at 37°C. As a control, the DCs were given the same doses of FITC-dextran and stored for 30 minutes at 4°C. After the incubation the DCs were washed and subjected to FACS analysis.

Cytokine Analysis. BDCA1+ DCs were cultured at a density of 2.5×10^5 cells/well in 96-well flat-bottom plates for 48 hours with 1,000 U/mL GM-CSF, 500 U/mL (hu) IL-4 in each of the media. At 24 hours, 500 ng/mL LPS or poly(I:C) (30μg/mL) were added. The supernatants were collected after 48 hours and immediately IL-12 (p40+p70) and IL-10 were determined by specific cytokine enzyme-linked immunosorbent assay (ELISA) kits (Bender MedSystems) according to the manufacturer's instructions. For the interferon gamma (IFN-γ) production of PBMCs, PBMCs were cultured at a density of 2.5×10^5 cells/well in HCM or ACM for 48 hours, and at 5.0×10^5 cells/well in autologous plasma for 12 hours. IFN-γ was determined by specific cytokine ELISA kits (Bender MedSystems).

Mixed Lymphocytes Reaction (MLR). BDCA+ DCs were cultured at a density of 1.0×10^5 cells/well in 96-well round-bottom plates (Falcon) containing HCM or ACM with GM-CSF and IL-4 for 48 hours. At 24 hours culture, immature DCs were induced to mature using LPS or poly(I:C) for an additional 24 hours. The allostimulatory capacity of irradiated DCs (3,000 Rad) was tested in a one-way MLR with normal 2×10^5cells/well allogeneic CD4+ lymphocytes (isolated from PBMCs using magnetic beads) under CCM. Cocultured cells were maintained for 7 days and the proliferation rate of the cells was measured using an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) Assay (CellTiter 96 aqueous one-solution cell proliferation assay; Promega, Madison, WI) according to the manufacturer's instructions. On carboxyfluorescein succinimidyl ester (CFSE) staining, cells were analyzed using a CellTrace CFSE Cell Proliferation Kit (Molecular Probes, Eugene, OR). The staining methods followed the manufacturer's protocol.

Immunoblotting. DCs were cultured at a density of 3.0×10^5 cells/well in 96-well flat-bottom plates (Corn-

Table 4. Phenotypic Difference of BDCA1+DCs Derived from Patients with Cirrhosis and Healthy Volunteers

			CD40	CD83	CD86	HLA-DR
Isolated DC	Healthy control (n=4)		5 ± 1.4	6 ± 2.2	14 ± 3.1	166 ± 52.2
	LC patients (n=4)		12 ± 16.1	4 ± 1.4	12 ± 3.4	195 ± 79.3
Mature DC	Healthy control (n=5)	HCM	131 ± 54	240 ± 25	201 ± 67	910 ± 121
		ACM	121 ± 37	*190 ± 33**	*170 ± 53**	783 ± 90
	LC patients (n=5)	HCM	139 ± 44	*154 ± 48†*	*169 ± 37†*	*691 ± 112†*
		ACM	124 ± 47	*125 ± 45‡*	*122 ± 11‡*	625 ± 160

The MFI are presented for each marker as the mean ± SD of healthy controls and patients with cirrhosis (isolated DC: Patients 6, 7, 8, 10 / mature DC: Patients 8, 9, 10, 11, 12).

*Value of $P < 0.05$ vs. DCs of healthy control cultured under HCM (Wilcoxon t test).

†Value of $P < 0.05$ vs. DCs of healthy control cultured under HCM (Mann-Whitney U test).

‡Value of $P < 0.05$ vs. DCs of LC patients cultured under HCM (Wilcoxon t test).

ing) containing 200 μL medium supplemented with GM-CSF and IL-4 for 24 hours and the DCs were stimulated by poly(I:C) for 1 hour. The DCs were harvested and lysed using CelLyticTM-M Mammalian Cell Lysis/Extraction Reagent (Sigma). The lysed cells were centrifuged to pellet the cellular debris. Thereafter, these protein concentrations were determined by a Modified Lowry Protein Assay Kit (Pierce, Rockford, IL). Equal amounts of protein were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to PVDF (Immun-Blot PVDF Membrane; Bio-Rad, Hercules CA). After washing and blocking, immunostaining was performed with rabbit polyclonal primary antibody (PI3K, phospho-PI3K, mTOR, p70 S6K, phospho-p70 S6K; Cell Signaling Technology, Beverly, MA), followed by incubation with a secondary antibody conjugated to horseradish peroxidase (HRP) (Sigma). Immunoreactive proteins were revealed with an ECL reagent (ECL advance; Amersham Biosciences, Little Chalfont, UK).

Oral Administration of BCAA to Patients with Advanced Cirrhosis and* Ex Vivo *Cytokine Production Assay. In the early morning we measured the fasting concentration of the plasma amino acids before and after oral administration of BCAA granules (30, 60, 120, 180 minutes) from healthy volunteers and patients with advanced cirrhosis. The BCAA granules: LIVACT (Ajinomoto Pharma, Tokyo, Japan) were composed of a mixture of valine, 1.144 g, leucine, 1.904 g, and isoleucine, 0.952 g. The concentrations of the plasma amino acids were measured by HPLC. We stimulated PBMCs from patients for 12 hours by LPS or poly(I:C) under autologous plasma, which was collected both before and after oral administration. After 12 hours we recovered the plasma and measured the IFN-γ by ELISA.

Statistical Analysis. The data were analyzed with analysis of variance (ANOVA) and multiple comparisons were performed with Dunnett's post-hoc procedure for the plasma aminogram. When two groups were analyzed, the differences between media were analyzed by the Wilcoxon t test. Frequencies of BDCA1+ DCs were compared between patient groups by the Mann-Whitney U test. All statistical analyses were performed with standard statistical software (SPSS 13.0 for Windows, Chicago, IL).

Results

Amino Acid Concentrations Similar to Those in Plasma of Patients with Advanced Cirrhosis Impaired the Maturation of Myeloid DCs from Healthy Controls. First we measured the cytokine production from PBMCs both under HCM and ACM. The IFN-γ production of PBMCs stimulated by poly(I:C) under ACM was significantly impaired (28.1 ± 7.3 pg/mL versus 16.7 ± 3.9 pg/mL; $P = 0.04$). Next, we cultured the BDCA+ DCs (purity >90%) for 48 hours under HCM and ACM and evaluated the phenotypes of DCs by flow cytometry. In ACM, the CD83 and CD86 expression of DCs was significantly impaired compared to that in HCM (Table 4). The HLA-DR expression had a tendency to decrease in ACM. This phenomenon was observed in MoDCs (Supporting Fig. 1). Next, The IL-12 production of BDCA+ DCs stimulated under ACM was significantly impaired (110.7 ± 8.6 pg/mL versus 79.9 ± 12.5 pg/mL; $P = 0.04$), although the IL-10 production of DCs was not different between HCM and ACM (31.0 ± 4.0 versus 32.4 ± 8.2; $P = 0.59$). Flow cytometric analysis revealed that the amount of FITC-dextran taken up by BDCA+ DC and MoDC did not differ between HCM and ACM (data not shown). The allostimulatory capacity of BDCA+ DCs cultured under ACM was significantly decreased as shown by the MTS assay (1.00 ± 0.15 versus 0.82 ± 0.13; $P = 0.04$; absorbance 490 nm), and this tendency was also confirmed by the CFSE assay.

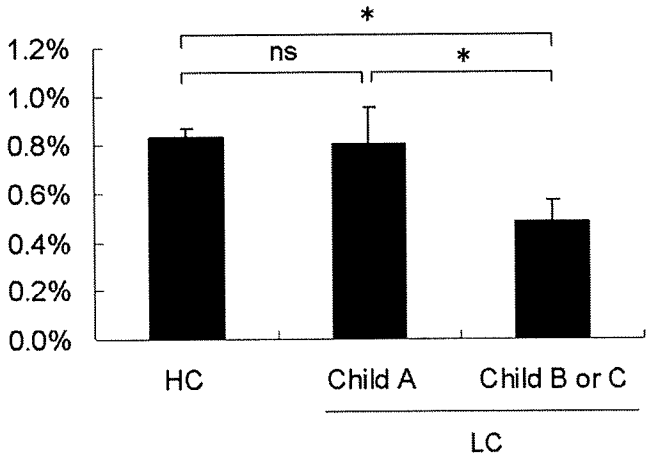

Fig. 1. The frequencies of DCs were significantly lower in the peripheral blood from patients with advanced cirrhosis compared with those from HC or early patients with cirrhosis. Percentages of BDCA+ DC in PBMCs were determined by flow cytometry. Significant differences in the percentages of DCs were observed between patients with advanced cirrhosis (Child-Pugh grade B or C: n = 10) and HC (n = 7). There was no difference between patients with Child-Pugh grade A (n = 7) and HC. Data are expressed as mean ± standard error of the mean (SEM).

Amino Acid Concentrations Similar to Those in Plasma of Patients with Advanced Cirrhosis Also Impaired the Maturation of Myeloid DCs from Patients with Cirrhosis. We first evaluated the frequency of BDCA+ DCs between HC and LC (Fig. 1). The frequencies of DCs were significantly lower in the peripheral blood from patients with advanced cirrhosis compared to those from HC or patients with early cirrhosis. Second, we determined the phenotype of BDCA1+ DCs from the LC before and after adding the stimulants. There was no difference regarding the mean fluorescence intensity (MFI) of isolated immature DCs expressing CD40, CD83, CD86, and HLA-DR between the HC and LC (Table 4). After adding the stimulants, the expressions of CD83 and HLA-DR by DCs from the LC were significantly decreased compared to those from the HC in both HCM and ACM (Table 4). The CD83 and CD86 expression of DCs was significantly impaired in ACM compared to that in HCM (Table 4).

Elevating the Concentration of BCAA Enhanced the IL-12 Production in BDCA+ DCs. As in the *in vivo* study, we confirmed that the plasma concentrations of BCAAs were significantly decreased and AAAs (except tryptophan) were increased along with the Child-Pugh grade (Table 1). Based on these data, to investigate which amino acid especially influenced the function of BDCA1+ DCs, we measured the cytokine production of DCs under HCM, ACM, and ACM supplemented with 800 nmol/mL of a single amino acid: valine, leucine, isoleucine, or AAAs. Interestingly, the IL-12 production of DCs stimulated under ACM plus valine or leucine was more increased than that under ACM, although there was no difference among ACM plus isoleucine, ACM plus AAAs, and ACM (Fig. 2A). Similar to the cytokine production, the allostimulatory capacity of DCs cultured under ACM plus valine or leucine had a tendency to be increased, as shown by the MTS assay (ACM: 0.71 ± 0.07, ACM plus valine: 0.88 ± 0.06; ACM plus leucine: 0.83 ± 0.03; absorbance 490 nm). Next, we determined the BDCA1+ DCs phenotype (CD14 and CD83) in CCM, BCAA-depleted, valine-depleted, leucine-depleted, and isoleucine-depleted media. In CCM, leucine-depleted and isoleucine-depleted media the DC phenotypes were similar (the percentages of CD83-positive cells were 33.7 ± 7.2%, 31.5 ± 5.4%, and 35.5 ± 7.9% for CCM, leucine-depleted, and isoleucine-depleted media, respectively). However, in BCAA-depleted and valine-depleted media, the CD83 expression of DCs was significantly impaired compared to that in CCM (BCAA-depleted media: 19.6 ± 3.0% and valine-depleted media 14.6 ± 1.8%; $P = 0.04$ versus CCM). After we cultured the DCs under depletion of valine for 2 days, we added valine to the medium and cultured the cells for an additional 24 hours. Then, the percentage of mature DCs was higher than that of valine-depleted media. Furthermore, to reflect more closely the actual environment of the living body, we induced DCs from LC to mature with either autologous plasma or autologous plasma supplemented with 100 nmol/mL valine for 12 hours. In all cases the DCs matured in the autologous plasma with valine had enhanced allostimulatory capacity and IL-12 production (Fig. 2B).

Amino Acid Concentration of Plasma in Patients with Advanced Cirrhosis Down-regulated the mTOR/S6K Signaling Pathway of BDCA1+ DCs. We hypothesized that the amino acid imbalance of the plasma in patients with advanced cirrhosis influence the mTOR/S6K signaling pathway of DCs and impaired their maturation. Under HCM with rapamycin, the percentage of CD14-/CD83+ mature DCs was higher than under HCM without rapamycin (Fig. 3A). BDCA+ DCs expressed similar levels of total PI3K, phospho-PI3K, mTOR, p70 S6K, and β-actin among all media. Interestingly, DCs cultured in ACM expressed lower levels of phospho-p70 S6K than those cultured in HCM (Fig. 3B). The expression of phospho-p70 S6K by DCs in ACM was partially recovered by adding 400 nmol/mL BCAA to the medium during stimulation. Isolated immature BDCA+ DCs expressed moderate levels of CD98 which modulate the amino acid transport functions and, after adding the stimulants, mature DC showed the up-regulation of CD98. There was no difference regarding

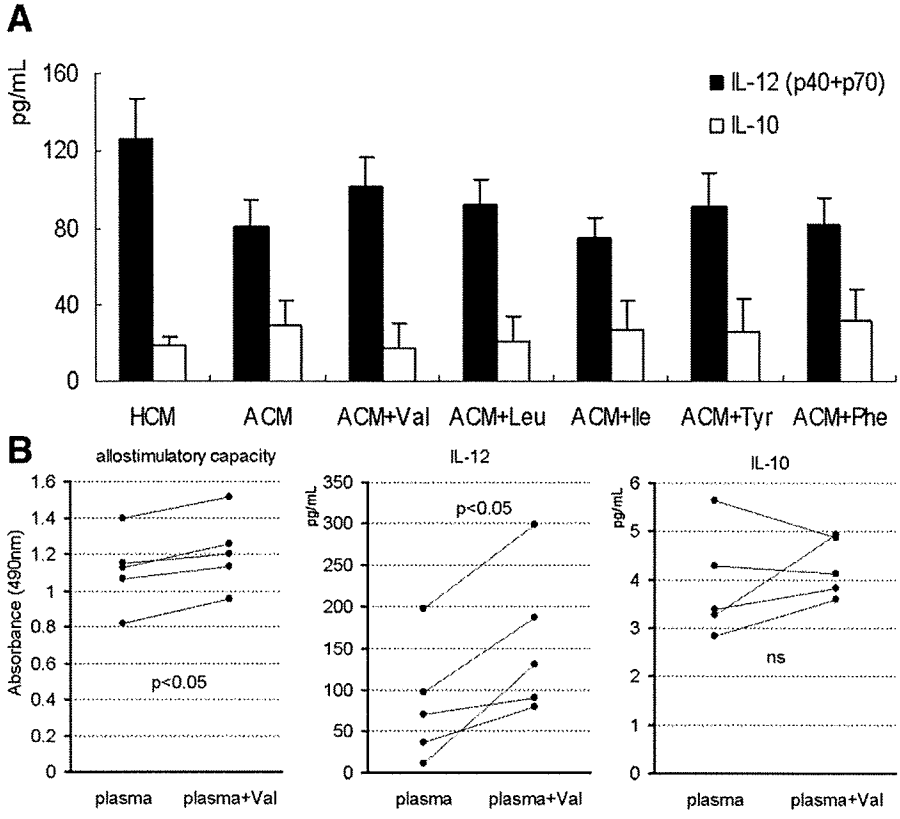

Fig. 2. Elevating the concentration of BCAAs enhanced the IL-12 production in BDCA1+ DCs. Isolated BDCA1+ DCs were cultured under HCM, ACM, and ACM supplemented with 800 nmol/mL single amino acid: valine, leucine, isoleucine, or AAAs. (A) After 48 hours the supernatants were assayed for cytokine concentrations. Mean ± SEM values from five different donors. (B) We induced BDCA1+ DCs from LC patients (Patients 1-5) to mature with either autologous plasma or autologous plasma supplemented with 100 nmol/mL valine for 12 hours. Supernatants were measured by ELISA. $P < 0.05$ (paired Student's t test, two-tailed).

the expression of CD98 between HCM and ACM (data not shown).

Oral Administration of BCAAs Enhanced the Production of IFN-γ by PBMCs from Patients with Advanced Cirrhosis Ex Vivo. Finally, we evaluated whether BCAAs have an effect on the immune response *ex vivo*. In healthy volunteers the concentration BCAAs of plasma was maximum 30 minutes after oral administration (Fig. 4A). Fischer's ratio increased from 4.78 ± 1.41 (standard deviation [SD]) to 13.39 ± 2.41 (SD). On the other hand, in the patients with advanced cirrhosis (Table 3: Patients 10-13), the concentration BCAAs of plasma was maximum 60 minutes after oral administration. Fischer's ratio increased from 1.37 ± 0.98 (SD) to 4.94 ± 0.99 (SD). AAAs decreased slowly during the following 3 hours. We stimulated PBMCs from the patients with advanced cirrhosis (Table 3: Patients 11-15) using either autologous plasma before and after 60 minutes oral administration. Interestingly, in all cases PBMCs stimulated by LPS in the latter had more IFN-γ production than the former (Fig. 4B).

Discussion

In this study we started by making two serum-free media (HCM and ACM) to be more representative of the human physiological environment and quantitatively measured the plasma amino acid profiles. First, we found that the amino acid imbalance of plasma in patients with advanced cirrhosis impaired the production of IFN-γ from PBMCs. IFN-γ is a dimerized soluble cytokine that is the only member of the type II class of interferons.[23] IFN-γ is secreted by Th1 cells, DCs, and NK cells. Although the commitment toward either the Th1 or the Th2 phenotype can be influenced by many signals active at the moment of naive Th cell priming, the levels of IL-12p70 (IL-12) produced by APC, especially DCs, are of major importance.[24,25] Therefore, we hypothesized that the impaired production of IFN-γ from PBMCs caused the dysfunction of DCs. Expectedly, the maturation and the IL-12 production of DCs were impaired in ACM. Furthermore, we confirmed that the allostimulatory capacity of DCs stimulated in ACM was impaired by MTS and CFSE assays. Previous studies have suggested an increase in IL-10 in cirrhosis and a potential link between high IL-10 and low HLA-DR expression in relation to immune dysfunction,[26] but in this study there was no difference in IL-10 secretion between DCs from ACM compared with HCM. Such differences were probably caused by (1) differences in the stimulation period of the immune cells (the former was *ex vivo*, this study was *in vitro*); (2) differences in the cell sources (the former was monocytes, this study was DCs); (3) other factors besides amino acids influence IL-10 production. Also in patients

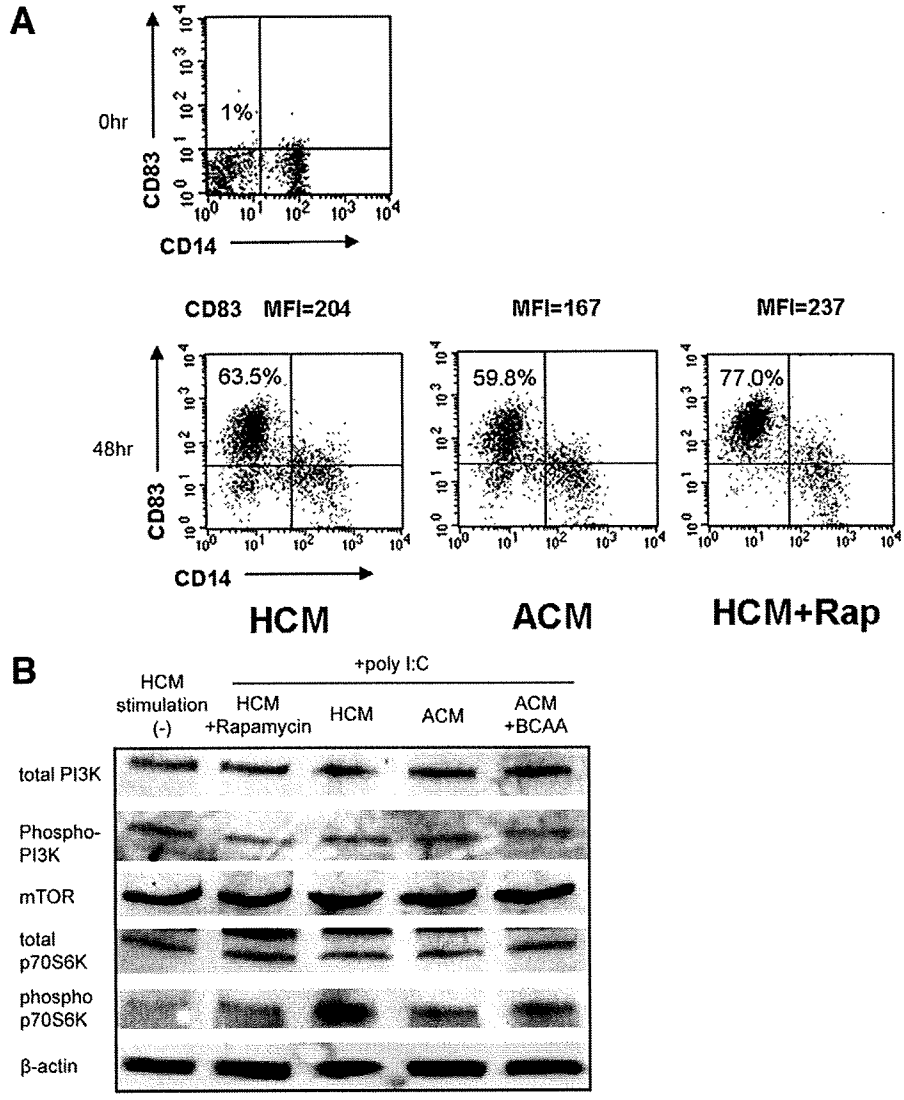

Fig. 3. Amino acid imbalance in plasma of patients with advanced cirrhosis down-regulated the mTOR/S6K signaling pathway of BDCA1+ DCs. (A) We stimulated BDCA1+ DCs under HCM, ACM, and HCM plus rapamycin (500 nM) for 24 hours with GM-CSF and IL-4, and exposed them to poly(I:C) for an additional 24 hours. We evaluated the phenotypes of DCs by flow cytometry. The percentages indicate the proportion of cells adopting the DC immunophenotype (CD14−/CD83+). (B) We cultured BDCA1+ DCs under HCM and ACM for 24 hours with GM-CSF and IL-4 and stimulated them with poly(I:C) for 1 hour. We also evaluated HCM plus rapamycin, and ACM plus BCAA. Equal amounts of protein were loaded and the levels of PI3K, phospho-PI3K, mTOR, p70 S6K, and phosho-p70 S6K were determined by Western blot analysis. (A,B) Data shown are representative of four independent experiments with cells from different donors.

with cirrhosis, the CD83 and CD86 expression of DCs stimulated under ACM was lower than that under HCM. When compared under the same medium, the CD83, CD86, and HLA-DR expressions of DCs from LC were lower than those from DCs of HC. To summarize these results, in advanced cirrhosis not only the DCs themselves but also the extracellular environments tend to impair the maturation of DCs.

Second, we examined which amino acids more strongly influences the function of DCs between HCM and ACM. We found that BCAA, especially valine and leucine, increased the BDCA+ DC allostimulatory capacity and IL-12 production. This confirms the findings of our previous study,[15] although the enhancement by a single amino acid was very subtle. To obtain greater enhancements, we may need to use combinations of other amino acids.

Concerning the mechanism that underlies these phenomena, we confirmed that the CD98 expression of DCs were not different between HCM and ACM. CD98 can regulate the expression and distribution of the light chains to modulate the amino acid transport functions. CD98hc is highly expressed on proliferating lymphocytes and on other rapidly growing cells.[27] Next, we examined whether the amino acid imbalance in the plasma of patients with advanced cirrhosis influenced the mTOR/S6K signaling pathway of the DCs. Recently, some studies reported the PI3K-mediated negative feedback regulation of IL-12 production in DCs,[28] and rapamycin-enhanced IL-12 production in LPS-stimulated DC.[29,30] In the present study, BDCA+ DCs stimulated in ACM impaired IL-12 production, even though the mTOR signaling was decreased. This paradox raises the possibility that the amino acid imbalance influences not only mTOR signaling but also other types of signaling such as GSK3 or NF-κB signaling. This issue should be evaluated in future studies.

Finally, we investigated whether elevating the level of plasma BCAAs enhances the immune response *ex vivo* in

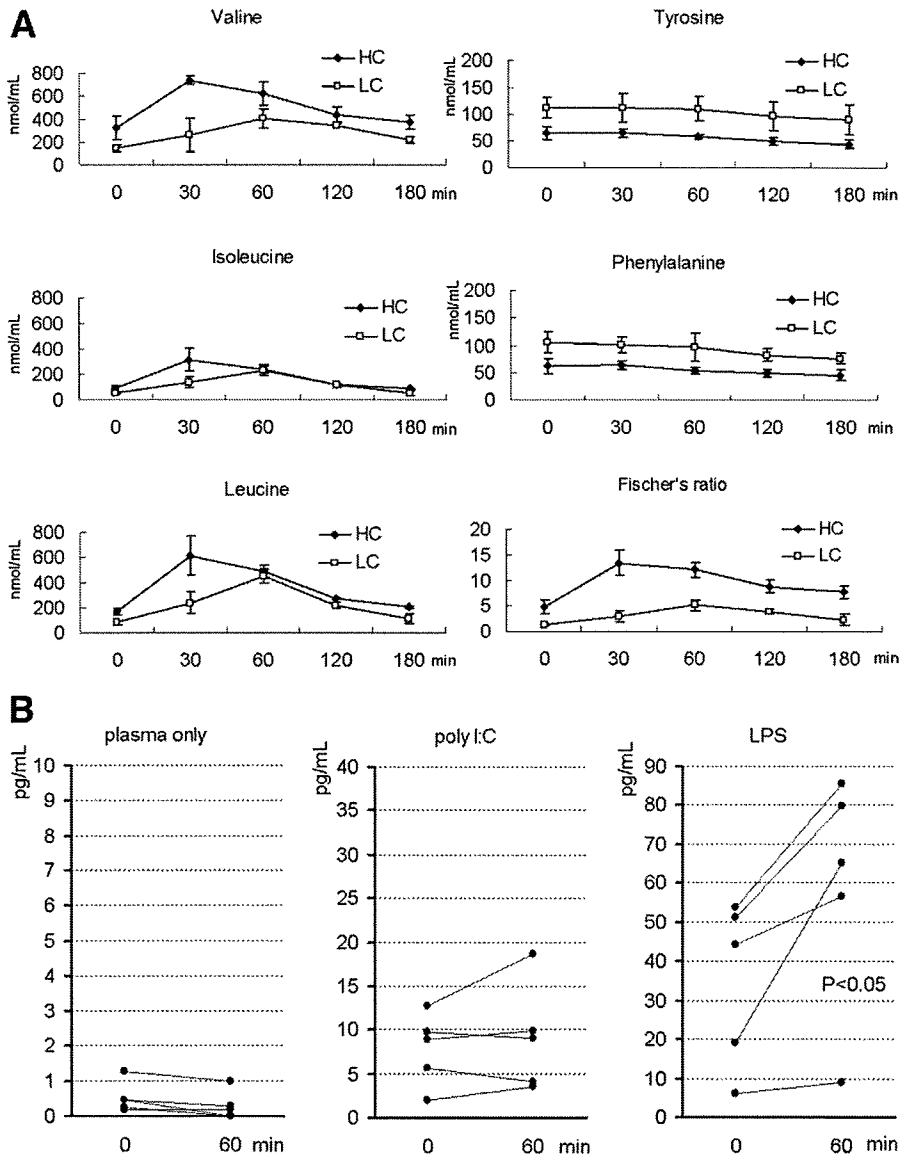

Fig. 4. Oral administration of BCAA granules enhanced the production of inflammatory cytokines from PBMCs stimulated by LPS *ex vivo*. (A) We analyzed the kinetics of the plasma amino acids after oral administration of BCAA granules. In the early morning while fasting, the concentrations of plasma amino acids were measured before and after oral administration of BCAA (30, 60, 120, 180 minutes). Mean ± SD values from three different HC and four patients with advanced cirrhosis (Patients 10-13). (B) We stimulated PBMCs from the patients using either autologous plasma before or after 60 minutes oral administration. After 12 hours we recovered the plasma and measured the IFN-γ by ELISA (Patients 11-15). $P < 0.05$ (paired Student's t test, two-tailed).

patients with advanced cirrhosis. BCAA granules have been used to effectively reverse the hypoalbuminemia and hepatic encephalopathy in patients with advanced cirrhosis.[31] In the preliminary investigation, we analyzed the kinetics of plasma amino acids after oral administration of BCAA granules. After oral administration, the BCAA concentration in plasma was maximal at 30 minutes in healthy volunteers. This was in contrast to patients with advanced cirrhosis, who had a slow increase in BCAA plasma concentrations that was maximal at 60 minutes. This difference was probably caused by the malabsorption of amino acids in the patients. In the *ex vivo* study, we could not use the medium to analyze the function of DCs of PBMCs because the concentration of the amino acids in medium influences the function. Thus, we stimulated cells in autologous plasma and analyzed the function over a short period of time. We found that oral administration of BCAAs enhanced the production of IFN-γ from PBMCs *ex vivo* in patients with advanced cirrhosis.

The results of this study still cannot be construed as conclusive evidence of a change in the functional clinical state in terms of lowering the risk of sepsis in cirrhosis or enabling consideration of such treatment for viral hepatitis. We need to perform a prospective, randomized, controlled trial in a well-characterized group of patients with appropriate immune mechanistic evaluation and determine the effects on the risk of sepsis in a longitudinal follow-up. In the present study we demonstrated at least that extracellular amino acids, especially BCAAs, influence the function of the immune system, and the amino acid imbalance in the plasma of patients with advanced cirrhosis impaired the maturation of DCs and the production of inflammatory cytokines from PBMCs or DCs.

In conclusion, the data from this study provide a rationale for future studies utilizing nutrition therapies that could be beneficial to immune function in patients with advanced cirrhosis.

References

1. Such J, Runyon BA. Spontaneous bacterial peritonitis. Clin Infect Dis 1998;27:669-674; quiz 675-676.
2. Morgan MY, Milsom JP, Sherlock S. Plasma ratio of valine, leucine and isoleucine to phenylalanine and tyrosine in liver disease. Gut 1978;19: 1068-1073.
3. Marchesini G, Bianchi G, Merli M, Amodio P, Panella C, Loguercio C, et al. Nutritional supplementation with branched-chain amino acids in advanced cirrhosis: a double-blind, randomized trial. Gastroenterology 2003; 124:1792-1801.
4. Nakaya Y, Okita K, Suzuki K, Moriwaki H, Kato A, Miwa Y, et al. BCAA-enriched snack improves nutritional state of cirrhosis. Nutrition 2007;23: 113-20.
5. Kobayashi M, Ikeda K, Arase Y, Suzuki Y, Suzuki F, Akuta N, et al. Inhibitory effect of branched-chain amino acid granules on progression of compensated liver cirrhosis due to hepatitis C virus. J Gastroenterol 2008; 43:63-70.
6. Muto Y, Sato S, Watanabe A, Moriwaki H, Suzuki K, Kato A, et al. Overweight and obesity increase the risk for liver cancer in patients with liver cirrhosis and long-term oral supplementation with branched-chain amino acid granules inhibits liver carcinogenesis in heavier patients with liver cirrhosis. Hepatol Res 2006;35:204-214.
7. Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, Lane WS, et al. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature 1994;369:756-758.
8. Dennis PB, Jaeschke A, Saitoh M, Fowler B, Kozma SC, Thomas G. Mammalian TOR: a homeostatic ATP sensor. Science 2001;294:1102-1105.
9. Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, et al. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell 2002;110:177-189.
10. Patti ME, Brambilla E, Luzi L, Landaker EJ, Kahn CR. Bidirectional modulation of insulin action by amino acids. J Clin Invest 1998;101:1519-1529.
11. Greiwe JS, Kwon G, McDaniel ML, Semenkovich CF. Leucine and insulin activate p70 S6 kinase through different pathways in human skeletal muscle. Am J Physiol Endocrinol Metab 2001;281:E466-E471.
12. Ijichi C, Matsumura T, Tsuji T, Eto Y. Branched-chain amino acids promote albumin synthesis in rat primary hepatocytes through the mTOR signal transduction system. Biochem Biophys Res Commun 2003;303:59-64.
13. Laviano A, Meguid MM, Inui A, Rossi-Fanelli F. Role of leucine in regulating food intake. Science 2006;313:1236-1238; author reply 1236-1238.
14. Calder PC. Branched-chain amino acids and immunity. J Nutr 2006;136: 288S–293S.
15. Kakazu E, Kanno N, Ueno Y, Shimosegawa T. Extracellular branched-chain amino acids, especially valine, regulate maturation and function of monocyte-derived dendritic cells. J Immunol 2007;179:7137-7146.
16. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245-252.
17. Zhou LJ, Tedder TF. Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. J Immunol 1995; 154:3821-3835.
18. Fiuza C, Salcedo M, Clemente G, Tellado JM. In vivo neutrophil dysfunction in cirrhotic patients with advanced liver disease. J Infect Dis 2000; 182:526-533.
19. Mookerjee RP, Stadlbauer V, Lidder S, Wright GA, Hodges SJ, Davies NA, et al. Neutrophil dysfunction in alcoholic hepatitis superimposed on cirrhosis is reversible and predicts the outcome. HEPATOLOGY 2007;46: 831-840.
20. Auffermann-Gretzinger S, Keeffe EB, Levy S. Impaired dendritic cell maturation in patients with chronic, but not resolved, hepatitis C virus infection. Blood 2001;97:3171-3176.
21. Ninomiya T, Akbar SM, Masumoto T, Horiike N, Onji M. Dendritic cells with immature phenotype and defective function in the peripheral blood from patients with hepatocellular carcinoma. J Hepatol 1999;31:323-331.
22. Kamath PS, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, et al. A model to predict survival in patients with end-stage liver disease. HEPATOLOGY 2001;33:464-470.
23. Gray PW, Goeddel DV. Structure of the human immune interferon gene. Nature 1982;298:859-863.
24. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 1996;17:138-146.
25. O'Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. Immunity 1998;8:275-283.
26. Berres ML, Schnyder B, Yagmur E, Inglis B, Stanzel S, Tischendorf JJ, et al. Longitudinal monocyte human leukocyte antigen-DR expression is a prognostic marker in critically ill patients with decompensated liver cirrhosis. Liver Int 2009;29:536-543.
27. Verrey F, Closs EI, Wagner CA, Palacin M, Endou H, Kanai Y. CATs and HATs: the SLC7 family of amino acid transporters. Pflugers Arch 2004; 447:532-542.
28. Fukao T, Tanabe M, Terauchi Y, Ota T, Matsuda S, Asano T, et al. PI3K-mediated negative feedback regulation of IL-12 production in DCs. Nat Immunol 2002;3:875-881.
29. Ohtani M, Nagai S, Kondo S, Mizuno S, Nakamura K, Tanabe M, et al. Mammalian target of rapamycin and glycogen synthase kinase 3 differentially regulate lipopolysaccharide-induced interleukin-12 production in dendritic cells. Blood 2008;112:635-643.
30. Weichhart T, Costantino G, Poglitsch M, Rosner M, Zeyda M, Stuhlmeier KM, et al. The TSC-mTOR signaling pathway regulates the innate inflammatory response. Immunity 2008;29:565-577.
31. Charlton M. Branched-chain amino acid enriched supplements as therapy for liver disease. J Nutr 2006;136:295S–298S.

J Gastroenterol
DOI 10.1007/s00535-009-0155-2

Strong CD8$^+$ T-cell responses against tumor-associated antigens prolong the recurrence-free interval after tumor treatment in patients with hepatocellular carcinoma

Kazumasa Hiroishi · Junichi Eguchi · Toshiyuki Baba · Tomoe Shimazaki · Shigeaki Ishii · Ayako Hiraide · Masashi Sakaki · Hiroyoshi Doi · Shojiro Uozumi · Risa Omori · Takuya Matsumura · Tatsuro Yanagawa · Takayoshi Ito · Michio Imawari

Received: 10 August 2009 / Accepted: 19 October 2009

Abstract
Aim We investigated whether tumor-specific CD8$^+$ T-cell responses affect tumor-free survival as well as the relationship between CD8$^+$ T-cell responses against tumor-associated antigens (TAAs) and the clinical course after tumor treatment in patients with hepatocellular carcinoma (HCC).
Methods Twenty patients with HCC that were treated by radiofrequency ablation or trans-catheter chemo-embolization (TACE) and in whom HCC was undetectable by ultrasonography, CT, and/or MRI 1 month after treatment were enrolled in the study. Before and after treatment for HCC, analyses of TAA (glypican-3, NY-ESO-1, and MAGE-1)-specific CD8$^+$ T-cell responses were evaluated with an interferon-γ enzyme-linked immunospot (ELISpot) assay using peripheral CD8$^+$ T-cells, monocytes, and 104 types of 20-mer synthetic peptide overlapping by 10 residues and spanning the entirety of the 3 TAAs.
Results Sixteen out of 20 patients (80%) showed a positive response ($\geq$10 TAA-specific cells/10^5 CD8$^+$ T-cells) before or after treatment. When we performed univariate analysis of prognostic factors for the tumor-free period in the 20 patients, platelet count, prothrombin time, and the number of TAA-specific CD8$^+$ T-cells after treatment were significant factors ($P = 0.027$, 0.030, and 0.004, respectively). In multivariate analysis, the magnitude of the TAA-specific CD8$^+$ T-cell response ($\geq$40 TAA-specific cells/10^5 CD8$^+$ T-cells) was the only significant prognostic factor for a prolonged tumor-free interval (hazard ratio 0.342, $P = 0.022$).
Conclusions Our results suggest that strong TAA-specific CD8$^+$ T-cell responses suppress the recurrence of HCC. Immunotherapy to induce TAA-specific cytotoxic T lymphocytes by means such as the use of peptide vaccines should be considered for clinical application in patients with HCC after local therapy.

Keywords Hepatocellular carcinoma · CD8$^+$ T-cell response · Cytotoxic T lymphocyte · ELISpot assay · Immunotherapy

K. Hiroishi · J. Eguchi · T. Baba · T. Shimazaki · S. Ishii · A. Hiraide · M. Sakaki · H. Doi · S. Uozumi · R. Omori · T. Matsumura · T. Yanagawa · T. Ito · M. Imawari (✉)
Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan
e-mail: imawari@med.showa-u.ac.jp

Introduction

There are about 500,000 new patients with hepatocellular carcinoma (HCC) per year worldwide. Although vaccination against hepatitis B virus (HBV) and interferon (IFN)-based therapy against hepatitis C virus (HCV) will presumably reduce the number of HCC patients in the future, the incidence of HCC is still increasing in Asia and Africa because of the previous prevalence of infection with the virus. Progress in treatments for HCC has improved the prognosis of patients with HCC. However, HCC is usually associated with cirrhosis and often recurs even after complete treatment of the tumors in the remaining part of the cirrhotic liver. Thus, there is a strong need for the development of a new intervention therapy that suppresses the occurrence or recurrence of HCC effectively and that has fewer side effects. Immunotherapy may be such a treatment and may be applicable to the clinical treatment of HCC. In fact, some clinical trials have been performed [1–3].

Cytotoxic T lymphocytes (CTLs) are thought to be potent effector cells against cancers. CTLs recognize specific antigens, and the induction of CTLs specific for tumor-associated antigen (TAA) is an attractive procedure

Published online: 20 November 2009

for tumor therapy. The MAGE-1 gene was first identified as encoding a tumor-specific antigen on MZ-2-MEL cells, a melanoma cell line, in 1991 [4]. MAGE-1 gene and protein can be detected in many cancer tissues, and three articles reported the expression of MAGE-1 in HCC as 30, 68, or 78%, respectively, in a Japanese population [5–7]. In gastrointestinal tumors, immunotherapy using both dendritic cells and MAGE peptides has been performed for patients with primary malignant melanoma of the esophagus, and this therapy was able to induce peptide-specific immune responses [8].

NY-ESO-1 antigen, a member of the cancer-testis antigen family, was initially identified by a serological analysis of recombinant cDNA expression cloning in an esophageal cancer patient [9]. NY-ESO-1 mRNA was detected in 24–37% of HCCs by reverse transcription-polymerase chain reaction [10, 11].

Glypican-3 (GPC3) consists of 580 amino acids and is a heparan sulfate proteoglycan with a potential role in the control of cell division. GPC3 mRNA was detected in 74.8% of HCC tissues, but only in 3.2% of normal liver tissues [12], and GPC3 protein was detected in 72% of HCCs, but not in normal tissue using GPC-specific antibody [13]. The GPC3 protein can also be detected in sera of 40–53% of patients with HCC [14, 15].

These three antigens are thought to be attractive targets for cancer immunotherapy because they are expressed only in tumor tissues and testis, but not in normal tissues other than testis. On the basis of previous reports, it is assumed that most HCCs would express at least one of the three TAAs. Therefore, monitoring immune responses against these TAAs might help in the development of HCC immunotherapy, such as TAA-based vaccination. In this study, we investigated how the magnitude of $CD8^+$ T-cell responses against these TAAs determined by an IFN-γ enzyme-linked immunospot (ELISpot) assay is related to other clinical data and the tumor-free interval in patients with HCC, in order to explore the clinical application of such a TAA-based immunotherapy.

Methods

Patients

Twenty patients who were diagnosed with HCC at Showa University Hospital between 2006 and 2008 were enrolled in the study. They met the following study criteria: (1) pathologically confirmed as having HCC or a lesion with characteristic imaging features of HCC based on ultrasonography, CT, and/or MRI; (2) liver function classed as Child-Pugh A or B; (3) no extrahepatic metastasis or vascular invasion; (4) no previous or simultaneous cancers other than HCC; and (5) an indication for treatment such as radiofrequency ablation (RFA) or trans-catheter chemo-embolization (TACE). RFA was performed by well-trained hepatologists using usual methods according to previous reports [16]. A 16-gauge cooled-tip ablation electrode (Covidien, Boulder, CO) was used in the procedure. TACE was performed by well-experienced hepatologists and radiologists. A microcatheter was inserted from the femoral artery to the artery feeding the HCC superselectively after conventional hepatic angiography, and then a segmental or subsegmental TACE procedure was performed using gelatin, lipiodol, and either epirubicin hydrochloride or cisplatin. All patients were followed every 1–3 months by ultrasonography, CT, and/or MRI to examine the appearance of new lesions in the liver or other organs. The recurrence-free interval was defined as the period from the month of HCC treatment to the month when a recurrent and/or metastatic HCC was first detected after treatment. Clinical data (platelet count, prothrombin time, serum AST, ALT, albumin, total bilirubin level, and AFP level) were collected 1–7 days before HCC treatment. Chronic hepatitis C was diagnosed on the basis of detectable HCV RNA in serum using the Amplicor assay (Roche Diagnostics, Tokyo, Japan). Informed consent was obtained from each patient included in this study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Ethical Committee of Showa University.

Synthetic peptides of TAA

Twenty-mer peptides overlapping by 10 residues and spanning the entire MAGE-1, NY-ESO-1, and GPC3 proteins were synthesized based on the amino acid sequences reported previously as PepSets™ and purchased from Mimotopes (Clayton South, Victoria, Australia). These peptides were >80% pure. A total of 30 MAGE-1, 17 NY-ESO-1, and 57 GPC3 peptides were synthesized, as shown in Table 1. A total of 10–11 TTA peptides were pooled in a mixture (total 10 mixtures) at a concentration of 10 μg/ml each.

Preparation of $CD8^+$ T cells and monocytes from patients with HCC

PBMCs were isolated from heparinized peripheral blood by gradient centrifugation using Ficoll-Paque (Pharmacia-LKB Biotechnology, Uppsala, Sweden). As reported previously, peripheral $CD8^+$ T-cells and monocytes were separated from PBMCs using CD8 microbeads (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany) and a Monocyte Isolation Kit II (Miltenyi Biotec), respectively [17]. These cells were isolated using an autoMACS™ Pro Separator (Miltenyi Biotec). The purity of the cells was >95% on flow cytometry (data not shown).

Table 1 Synthetic peptides and peptide mixtures used in this study

Tumor-associated antigen	Peptide	Amino acid sequence
Glypican-3	GL1	1–20
	GL2	11–30
	GL3	21–40
	⋮	⋮
	GL57	561–580
MAGE-1	MG-1	1–20
	⋮	⋮
	MG-30	290–309
NY-ESO-1	NY-1	1–20
	⋮	⋮
	NY-17	161–180

Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix 6	Mix 7	Mix 8	Mix 9	Mix 10
GL1	GL2	GL3	GL4	GL5	GL6	GL7	GL8	GL9	GL10
GL11	GL12	GL13	GL14	GL15	GL16	GL17	GL18	GL19	GL20
GL21	GL22	GL23	GL24	GL25	GL26	GL27	GL28	GL29	GL30
GL31	GL32	GL33	GL34	GL35	GL36	GL37	GL38	GL39	GL40
GL41	GL42	GL43	GL44	GL45	GL46	GL47	GL48	GL49	GL50
GL51	GL52	GL53	GL54	GL55	GL56	GL57	MG-1	MG-2	MG-3
MG-4	MG-5	MG-6	MG-7	MG-8	MG-9	MG-10	MG-11	MG-12	MG-13
MG-14	MG-15	MG-16	MG-17	MG-18	MG-19	MG-20	MG-21	MG-22	MG-23
MG-24	MG-25	MG-26	MG-27	MG-28	MG-29	MG-30	NY-1	NY-2	NY-3
NY-4	NY-5	NY-6	NY-7	NY-8	NY-9	NY-10	NY-11	NY-12	NY-13
NY-14	NY-15	NY-16	NY-17	–	–	–	–	–	–

IFN-γ ELISpot assay

The ELISpot assay was performed using an IFN-γ ELISpot assay kit (Mabtech AB, Stockholm, Sweden) as previously described [17]. Briefly, a 96-well microtiter plate with a nitrocellulose membrane bottom (Millititer; Millipore, Bedford, MA) was coated with 100 μl anti-IFN-γ monoclonal antibody at a concentration of 15 μg/ml in phosphate-buffered saline (PBS) overnight at 4°C. Unbound antibody was removed by washing 6 times in Hanks' balanced saline solution. After blocking with AIM-V medium (Invitrogen Japan, Tokyo, Japan) containing 10% fetal bovine serum, 1×10^5 $CD8^+$ T-cells, 1×10^4 autologous monocytes, and a TAA peptide mixture at 10 μg/ml of each peptide were placed and incubated in duplicate in 100 μl AIM-V medium at 37°C in a humid atmosphere with 5% CO_2. After incubation for 18 h, the cells were removed by washing the plate 8 times with PBS. Next, 100 μl of biotin-conjugated monoclonal antibody was added to each well, and the plates were incubated further for 2 h at room temperature. Wells were washed 5 times with PBS and incubated with 100 μl streptavidin–alkaline phosphatase for 2 h. Unbound antibodies were removed by washing 6 times with PBS. Then, 100 μl of alkaline phosphatase substrate (Bio-Rad Laboratories, Richmond, CA) was added to each well and incubated until dark spots emerged. Color development was stopped by washing 3 times with water, and the plates were allowed to dry. Using an ELISpot reader (KS ELISPOT compact; Carl Zeiss, Oberkochen, Germany), the number of spot-forming cells (SFCs) per well was counted. Numbers of TAA-specific SFCs for each peptide mixture were calculated by subtracting the mean number of SFCs of 2 control wells (without stimulus) from the mean number of SFCs of 2 wells stimulated by TAA antigens. An SFC number was calculated for each patient as the sum of SFCs in each peptide mixture. ELISpot assays were performed before and 3–7 days after treatment. When TAA-specific $CD8^+$ T-cell responses were analyzed in 10 normal subjects, we were unable to detect any responses against TAA peptides in the ELISpot assay (data not shown).

Statistical analyses

The relationship between the number of TAA-specific $CD8^+$ T-cells and the recurrence-free period was analyzed

using a parametric survival model. The log-rank test was used to compare recurrence-free data for 2 groups. The effects of multiple explanatory variables on recurrence-free interval were analyzed using a Cox proportional hazards model. Statistical analyses were performed using the statistical software JMP version 5 (SAS Institute Inc., Cary, NC). Differences were considered as significant when the P value was less than 0.05.

Results

TAA-specific CD8$^+$ T-cells were detected by ELISpot assay before and after HCC treatment in most HCC patients

The characteristics of the 20 patients enrolled in this study are shown in Table 2. The 20 patients had no HCC detected by ultrasonography, enhanced CT, and/or MRI 1 month after treatment for HCC. In those patients with HCCs who had up to 3 HCCs and in whom the diameter of each lesion was 3 cm or less, the treatment was usually RFA; the remaining patients were treated by TACE. However, in a few patients (patients 2 and 5) in whom the diameter of each lesion was less than 3 cm, the physician in charge of the patient selected TACE because they could not deny the existence of more lesions that were undetectable by conventional enhanced CT. The clinical courses of the patients were followed for 3–29 months after therapy for HCC. The ELISpot assay was performed to detect CD8$^+$ T-cell responses to TAAs before and 3–7 days after treatment. The data are shown in Table 3 as SFCs (total count of TAA-specific CD8$^+$ T-cells/1 $\times$ 10^5 CD8$^+$ T-cells). Sixteen out of 20 patients (80%) showed a positive response (10 or more SFCs) for TAA peptides either before and/or after treatment. The numbers of SFCs (mean $\pm$ SD) before and after therapy were 33.8 $\pm$ 51.4 (0–161, median 16.5) and 32.9 $\pm$ 34.7 (0–130, median 23.0), respectively. Of the 20 patients, 5 (25%) and 7 (35%) showed a high TAA-specific immune response (40 or more SFCs) before and after treatment, respectively.

When we analyzed the TAA peptides recognized by CD8$^+$ T-cells, we occasionally observed that different peptide mixtures were identified as positive before and after HCC treatment (data not shown).

Change in TAA-specific CD8$^+$ T-cell response induced by HCC treatment does not correlate with recurrence-free period

The number of SFCs increased in 11 of 20 (55%) patients after treatment. In these patients, TAA-specific CTLs might have been induced by the treatment. There were no significant differences between the increase in TAA-specific CD8$^+$ T-cell response induced by the treatment and either therapeutic procedure, laboratory data, or background of the patients (data not shown). The increase in TAA-specific CTLs after treatment did not predict a better prognosis of HCC.

Table 2 Characteristics of HCC patients before HCC treatment

	$n = 20$	Median
Age (years)[a]	68.8 $\pm$ 9.4	73.0
Gender		
M	11	
F	9	
AST (IU/l)[a]	70 $\pm$ 49	52
ALT (IU/l)[a]	63 $\pm$ 43	54
PLT ($\times 10^4/\mu$l)[a]	9.8 $\pm$ 5.3	8.5
PT (%)[a]	81 $\pm$ 11	78
Alb (g/dl)[a]	3.5 $\pm$ 0.4	3.4
T-Bil (mg/dl)[a]	0.9 $\pm$ 0.4	0.9
AFP (ng/ml)[a]	86 $\pm$ 157	16
Virus		
HCV	17	
NBNC	3	
Child-Pugh class		
A	12	
B	8	
HCC size (mm)[a]	23 $\pm$ 8	23
No. HCCs		
1	9	
2	4	
3	7	
>3	0	
Treatment		
RFA	13	
TACE	5	
RFA + TACE	2	

NBNC Negative for neither HBV nor HCV infection, *RFA* radiofrequency ablation, *TACE* trans-catheter chemo-embolization

[a] Results are shown as mean $\pm$ SD

Platelet count, prothrombin time, and the magnitude of TAA-specific immune response after treatment correlate with the recurrence-free period by univariate analysis

When we analyzed the relationship between TAA-specific SFCs detected by the ELISpot assay or other clinical variates and the HCC-free interval using a parametric survival model, we found that platelet count, prothrombin time, and the TAA-specific CD8$^+$ T-cell response after treatment significantly correlated with the HCC-free interval

Table 3 Results of IFN-γ ELISpot assay in patients in whom HCCs were not detected after therapy

Patient no.	SFC before treatment (/10^5 $CD8^+$ T-cells)	SFC after treatment (/10^5 $CD8^+$ T-cells)	Recurrence-free interval (month)
1	0	0	5
2	15	31	10
3	12	15	5
4	159	130	26
5	58	4	12
6	5	99	29[a]
7	15	17	7
8	20	41	7
9	135	9	12
10	1	6	3
11	8	9	6
12	10	57	15
13	34	42	13[a]
14	6	4	12[a]
15	23	8	9
16	59	37	12
17	12	29	23
18	161	72	24
19	18	4	15
20	25	44	23[a]

SFC Spot-forming cells

[a] These patients had no recurrence detected by ultrasonography, enhanced CT, and/or MRI after treatment

($P = 0.005$, 0.007, and 0.001, respectively). When univariate analysis of prognostic factors for the HCC-free interval was performed, only platelet count ($P = 0.027$; Fig. 1a), prothrombin time ($P = 0.030$; Fig. 1b), and the number of SFCs after treatment ($P = 0.004$; Fig. 1c) were found to be significant. Child-Pugh class A tended to prolong the HCC-free interval, although this was not significant ($P = 0.066$). The other factors, including the number of SFCs before treatment ($P = 0.407$), ALT level ($P = 0.644$), albumin level ($P = 0.488$), total bilirubin level ($P = 0.340$), HCC size ($P = 0.756$), HCC number ($P = 0.486$), and the procedure used for HCC treatment (RFA or TACE, $P = 0.481$), did not affect HCC-free survival, as confirmed by the log-rank test.

Multivariate analysis shows that the magnitude of TAA-specific $CD8^+$ T-cell responses correlates with the HCC-free interval after treatment in patients who have no detectable HCC after therapy

In a further analysis of the 20 patients with HCC who were treated by RFA or TACE and in whom no HCC was detectable 1 month after treatment, we performed multivariate analysis using a Cox proportional hazards model. On multivariate analysis, only the magnitude of TAA-specific $CD8^+$ T-cell responses (≥40 TAA-specific cells/10^5 $CD8^+$ T-cells) was the only significant prognostic factor for a prolonged tumor-free period after treatment

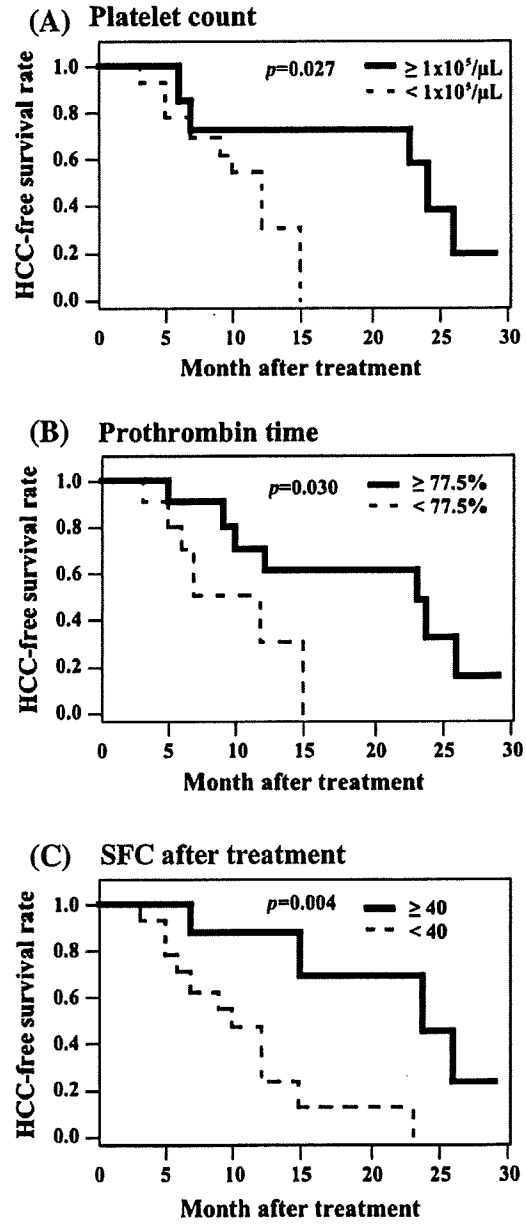

Fig. 1 Kaplan–Meier curves of HCC-free survival rate. In univariate analysis, platelet count, prothrombin time, and the tumor-associated antigen-specific $CD8^+$ T-cell response were found to be prognostic factors for the HCC-free period after treatment. Kaplan–Meier curves representing the relationship between month after treatment (HCC-free interval) and HCC-free survival rate were grouped by **a** platelet count, **b** prothrombin time, and **c** spot-forming cells (*SFCs*) specific for tumor-associated antigens after treatment

Table 4 Multivariate analyses of prognostic factors for tumor-free interval

Variable	Hazard ratio	95% Confidence limit	*P* value
Platelet count			
$\geq 1 \times 10^5/\mu L$	0.916	0.326–2.020	0.843
$<1 \times 10^5/\mu L$	1.000		
Prothrombin time			
≥77.5%	0.455	0.094–1.390	0.177
<77.5%	1.000		
Child-Pugh class			
A	1.464	0.539–6.813	0.493
B	1.000		
Spot-forming cells after treatment			
≥40	0.342	0.079–0.866	0.022
<40	1.000		

(hazard ratio 0.342, $P = 0.022$), as shown in Table 4. Therefore, the results suggest that TAA-specific CTLs detected after treatment are able to suppress the occurrence or recurrence of HCC in patients with no detectable HCCs after treatment.

Discussion

To determine whether TAA-specific CTLs suppress the occurrence or recurrence of HCC, we investigated the relationship between the magnitude of TAA-specific $CD8^+$ T-cell responses and the HCC-free interval in patients who had no detectable viable HCC 1 month after treatment for HCC. We found that potent TAA-specific $CD8^+$ T-cell responses, as observed 1 month after treatment for HCC, led to a prolonged HCC-free interval.

An HLA-A24-restricted MAGE-1 peptide-specific CTL line was established in a patient with metastatic melanoma [18], and an NY-ESO-1 DNA vaccine induced both antigen-specific effector $CD4^+$ and/or $CD8^+$ T-cell responses in most patients who did not show detectable pre-vaccination immune responses [19]. In addition, HLA-A2- and HLA-A24-restricted GPC3-derived peptide vaccine induced specific CTLs in mice [20]. In this study, we selected GPC3, MAGE-1, and NY-ESO-1 to monitor antigen-specific $CD8^+$ T-cell responses against HCC because they had been reported to be expressed commonly and frequently in HCC tissues [7, 11–13], and thus the combination of these TAAs would cover most HCCs. Among the 20 patients enrolled in the present study, 16 (80%) showed positive $CD8^+$ T-cell responses (10 or more SFCs) against the TAAs before and/or after the treatment. Although we did not examine the expression of TAAs in the HCC tissues, it would be expected that at least one of these three TAAs will be expressed in HCCs in patients who have a positive $CD8^+$ T-cell response against TAAs.

In patient 10, HCC recurrence was detected 3 months after treatment. Insufficient treatment or the pre-existence of intrahepatic metastases might be considered in a patient in whom HCCs are undetectable 1 month after treatment, but are detected within a few months after treatment. We expected that TAA-specific CTLs induced by treatment would suppress the development of a small HCC, which is not easily detected by conventional methods of examination. Thus, we enrolled and analyzed all patients in whom no HCC was detectable by ultrasonography, CT, and/or MRI 1 month after treatment, even if a recurrent or metastatic HCC was detected within a few months after treatment.

It is of interest whether tumor destruction by local HCC treatment would induce immune responses against HCCs. Apoptotic tumor cells are capable of inducing tumor-specific immune responses [21]. Dendritic cells, representing antigen-presenting cells, around damaged tumor cells take up tumor antigen released from the tumor cells and then migrate into draining lymph nodes [22]. There, they mature and stimulate tumor-specific helper T-cells and CTLs. In turn, the effector cells migrate into the tumor tissue and attack the tumor cells [23]. Tumor-specific immune responses were induced by a combination of direct dendritic cell injections into the HCC and radiation therapy that might induce tumor destruction [3]. When we compared TAA-specific $CD8^+$ T-cell responses before HCC treatment and those after treatment, about half of the patients (55%) showed an increased frequency of TAA-specific $CD8^+$ T-cells, which might have been induced by the treatment. However, the increase in TAA-specific CTLs did not affect the recurrence-free interval. Rather, it was the magnitude of TAA-specific $CD8^+$ T-cell responses after the treatment itself that affected the recurrence-free interval. Even if the frequency of these CTLs seemed to be decreased after treatment, they might infiltrate the liver. Furthermore, new CTLs other than pre-existing CTLs might be induced by the treatment because many TAA peptides recognized by CTLs were different between before and after the treatment. Although some patients showed a potent TAA-specific $CD8^+$ T-cell response before treatment, SFC before treatment did not correlate with the recurrence-free interval. We believe that TAA-specific CTLs are not able to control a large tumor burden by itself. As HCCs enlarge, they may secret immune suppressive factors such as TGF-β [24] and/or IL-10 [25] and modify gene expression of TAAs [26]. We speculate that TAA-specific CTLs detected after the treatment, but not detected before the treatment may be able to control HCCs. Otherwise, TAA-specific CTLs detected before the

treatment may be able to destroy a small HCC that was not detected by conventional examinations.

The ELISpot assay is a convenient means of detecting antigen-specific $CD8^+$ T-cells in a variety of diseases. We have detected HCV-specific $CD8^+$ T-cell responses in patients with acute HCV infection using this method and identified 6 new epitopes within the HCV protein [17]. In fact, we identified a novel GPC3-specific CTL epitope using this method (unpublished observation). At present, we are trying to identify more CTL epitopes among these TAAs that will be used as cancer vaccines.

In this study, we used peptide mixtures to stimulate $CD8^+$ T-cells. This procedure may mask responses to individual peptides because a peptide that interacts only weakly with HLA molecules is unable to attach to the molecule if the mixture contains 1 peptide with a high affinity for the same molecule. However, such a weak peptide would not contribute to tumor immune responses because of its weak interaction with the HLA molecules. Thus, we ignored this issue in this study.

Recurrence and multicentric carcinogenesis are major factors in determining the prognosis of HCC, and several treatments have been tried for the prevention of recurrence. IFN therapy [27, 28], treatment with acyclic retinoid therapy [29, 30], and adoptive immunotherapy [31] have been reported as effective in suppressing HCC recurrence. Preoperative hepatic function influenced early HCC recurrence in patients in whom small HCCs were resected [32]. This is consistent with our result that prothrombin time, reflecting hepatic function, affected the recurrence-free interval in the univariate analysis. In our study, higher platelet counts also contributed to a longer recurrence-free interval in the univariate analysis. In the multivariate analysis, however, only the magnitude of TAA-specific $CD8^+$ T-cell responses remained as an independent factor contributing to a longer recurrence-free interval.

Although the size and number of HCCs were reported to affect the period of HCC-free survival (recurrence) in patients with HCC treated by hepatic resection [33], they are not significant factors affecting the recurrence-free interval. Further investigation, such as the accumulation of analyses of HCC patients, is needed to clarify this issue. Sixteen out of 20 patients without detectable HCC 1 month after treatment had recurrent or metastatic HCCs during the observation period in this study. Our results suggest that the maintenance of strong TAA-specific $CD8^+$ T-cell responses for a long period may lead to a longer recurrence-free state. A long-term observation of TAA-specific immune responses should also be performed in any future study.

The results of our study suggest that strong TAA-specific $CD8^+$ T-cell responses would suppress HCC recurrence in patients with HCC who are treated by RFA or TACE and in whom any HCC is undetectable by ultrasonography, CT, and/or MRI 1 month after treatment. Since recurrence and intrahepatic metastasis are major risk factors influencing the prognosis of patients with HCC, immunotherapy to induce TAA-specific $CD8^+$ T-cells, such as a peptide vaccine, should be considered for clinical application in patients with HCC after local therapy.

Acknowledgments This study was supported in part by a grant from the Ministry of Health, Labor and Welfare of Japan (Kazumasa Hiroishi, Michio Imawari); a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Kazumasa Hiroishi); and a grant for the High-Technology Research Center Project from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Michio Imawari).

References

1. Palmer DH, Midgley RS, Mirza N, Torr EE, Ahmed F, Steele JC, et al. A phase II study of adoptive immunotherapy using dendritic cells pulsed with tumor lysate in patients with hepatocellular carcinoma. Hepatology. 2009;49:124–32.
2. Lee WC, Wang HC, Hung CF, Huang PF, Lia CR, Chen MF. Vaccination of advanced hepatocellular carcinoma patients with tumor lysate-pulsed dendritic cells: a clinical trial. J Immunother. 2005;28:496–504.
3. Chi KH, Liu SJ, Li CP, Kuo HP, Wang YS, Chao Y, et al. Combination of conformal radiotherapy and intratumoral injection of adoptive dendritic cell immunotherapy in refractory hepatoma. J Immunother. 2005;28:129–35.
4. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science. 1991;254:1643–7.
5. Suzuki K, Tsujitani S, Konishi I, Yamaguchi Y, Hirooka Y, Kaibara N. Expression of MAGE genes and survival in patients with hepatocellular carcinoma. Int J Oncol. 1999;15:1227–32.
6. Tahara K, Mori M, Sadanaga N, Sakamoto Y, Kitano S, Makuuchi M. Expression of the MAGE gene family in human hepatocellular carcinoma. Cancer. 1999;85:1234–40.
7. Kariyama K, Higashi T, Kobayashi Y, Nouso K, Nakatsukasa H, Yamano T, et al. Expression of MAGE-1 and -3 genes and gene products in human hepatocellular carcinoma. Br J Cancer. 1999;81:1080–7.
8. Ueda Y, Shimizu K, Itoh T, Fuji N, Naito K, Shiozaki A, et al. Induction of peptide-specific immune response in patients with primary malignant melanoma of the esophagus after immunotherapy using dendritic cells pulsed with MAGE peptides. Jpn J Clin Oncol. 2007;37:140–5.
9. Chen YT, Scanlan MJ, Sahin U, Tureci O, Gure AO, Tsang S, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. Proc Natl Acad Sci USA. 1997;94:1914–8.
10. Korangy F, Ormandy LA, Bleck JS, Klempnauer J, Wilkens L, Manns MP, et al. Spontaneous tumor-specific humoral and cellular immune responses to NY-ESO-1 in hepatocellular carcinoma. Clin Cancer Res. 2004;10:4332–41.
11. Chen CH, Chen GJ, Lee HS, Huang GT, Yang PM, Tsai LJ, et al. Expressions of cancer-testis antigens in human hepatocellular carcinomas. Cancer Lett. 2001;164:189–95.
12. Hsu HC, Cheng W, Lai PL. Cloning and expression of a developmentally regulated transcript MXR7 in hepatocellular

carcinoma: biological significance and temporospatial distribution. Cancer Res. 1997;57:5179–84.
13. Capurro M, Wanless IR, Sherman M, Deboer G, Shi W, Miyoshi E, et al. Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology. 2003;125:89–97.
14. Nakatsura T, Yoshitake Y, Senju S, Monji M, Komori H, Motomura Y, et al. Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun. 2003;306:16–25.
15. Hippo Y, Watanabe K, Watanabe A, Midorikawa Y, Yamamoto S, Ihara S, et al. Identification of soluble NH2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. Cancer Res. 2004;64:2418–23.
16. Shiina S, Teratani T, Obi S, Sato S, Tateishi R, Fujishima T, et al. A randomized controlled trial of radiofrequency ablation with ethanol injection for small hepatocellular carcinoma. Gastroenterology. 2005;129:122–30.
17. Doi H, Hiroishi K, Shimazaki T, Eguchi J, Baba T, Ito T, et al. Magnitude of CD8 T-cell responses against hepatitis C virus and severity of hepatitis do not necessarily determine outcomes in acute hepatitis C virus infection. Hepatol Res. 2009;39:256–65.
18. Akiyama Y, Maruyama K, Tai S, Komiyama M, Iizuka A, Takikawa M, et al. Characterization of a MAGE-1-derived HLA-A24 epitope-specific CTL line from a Japanese metastatic melanoma patient. Anticancer Res. 2009;29:647–55.
19. Gnjatic S, Altorki NK, Tang DN, Tu SM, Kundra V, Ritter G, et al. NY-ESO-1 DNA vaccine induces T-cell responses that are suppressed by regulatory T cells. Clin Cancer Res. 2009; 15:2130–9.
20. Motomura Y, Ikuta Y, Kuronuma T, Komori H, Ito M, Tsuchihara M, et al. HLA-A2 and -A24-restricted glypican-3-derived peptide vaccine induces specific CTLs: preclinical study using mice. Int J Oncol. 2008;32:985–90.
21. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature. 1998;392:86–9.
22. Onaitis M, Kalady MF, Pruitt S, Tyler DS. Dendritic cell gene therapy. Surg Oncol Clin N Am. 2002;11:645–60.
23. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392:245–52.
24. Gastl G, Plante M, Finstad CL, Wong GY, Federici MG, Bander NH, et al. High IL-6 levels in ascitic fluid correlate with reactive thrombocytosis in patients with epithelial ovarian cancer. Br J Haematol. 1993;83:433–41.
25. Hersh EM, Stopeck AT. Advances in the biological therapy and gene therapy of malignant disease. Clin Cancer Res. 1997; 3:2623–9.
26. Cormier JN, Panelli MC, Hackett JA, Bettinotti MP, Mixon A, Wunderlich J, et al. Natural variation of the expression of HLA and endogenous antigen modulates CTL recognition in an in vitro melanoma model. Int J Cancer. 1999;80:781–90.
27. Ikeda K, Arase Y, Saitoh S, Kobayashi M, Suzuki Y, Suzuki F, et al. Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor-A prospective randomized study of hepatitis C virus-related liver cancer. Hepatology. 2000;32:228–32.
28. Kubo S, Nishiguchi S, Hirohashi K, Tanaka H, Shuto T, Yamazaki O, et al. Effects of long-term postoperative interferon-alpha therapy on intrahepatic recurrence after resection of hepatitis C virus-related hepatocellular carcinoma. A randomized, controlled trial. Ann Intern Med. 2001;134:963–7.
29. Muto Y, Moriwaki H, Ninomiya M, Adachi S, Saito A, Takasaki KT, et al. Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma. Hepatoma Prevention Study Group. N Engl J Med. 1996; 334:1561–7.
30. Muto Y, Moriwaki H, Saito A. Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma. N Engl J Med. 1999;340:1046–7.
31. Takayama T, Sekine T, Makuuchi M, Yamasaki S, Kosuge T, Yamamoto J, et al. Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: a randomised trial. Lancet. 2000;356:802–7.
32. Kaibori M, Ishizaki M, Saito T, Matsui K, Kwon AH, Kamiyama Y. Risk factors and outcome of early recurrence after resection of small hepatocellular carcinomas. Am J Surg. 2009;198:39–45.
33. Maeda T, Shimada M, Harimoto N, Tsujita E, Aishima S, Tanaka S, et al. Prognosis of early hepatocellular carcinoma after hepatic resection. Hepatogastroenterology. 2008;55:1428–32.

Journal of Medical Virology 81:619–627 (2009)

Infection of B Cells With Hepatitis C Virus for the Development of Lymphoproliferative Disorders in Patients With Chronic Hepatitis C

Momoko Inokuchi,[1] Takayoshi Ito,[1]* Manabu Uchikoshi,[1] Yuu Shimozuma,[1] Kenichi Morikawa,[1] Hisako Nozawa,[1] Tomoe Shimazaki,[1] Kazumasa Hiroishi,[1] Yuzo Miyakawa,[2] and Michio Imawari[1]

[1]*Department of Gastroenterology, Showa University School of Medicine, Tokyo, Japan*
[2]*Miyakawa Memorial Research Foundation, Tokyo, Japan*

Infection with hepatitis C virus (HCV) is associated with lymphoproliferative disorders, represented by essential mixed cryoglobulinemia and B-cell non-Hodgkin's lymphoma, but the pathogenic mechanism remains obscure. HCV may infect B cells or interact with their cell surface receptors, and induce lymphoproliferation. The influence of HCV infection of B cells on the development of lymphoproliferative disorders was evaluated in 75 patients with persistent HCV infection. HCV infection was more prevalent (63% vs. 16%, 14%, or 17% $P < 0.05$ for each), and HCV RNA levels were higher (3.35 ± 3.85 vs. 1.75 ± 2.52, 2.15 ± 2.94 or 2.10 ± 2.90 log copies/100 ng, $P < 0.01$ for each) in B cells than $CD4^+$, $CD8^+$ T cells or other cells. Negative-strand HCV RNA, as a marker of viral replication, was detected in B cells from four of the 75 (5%) patients. Markers for lymphoproliferative disorders were more frequent in the 50 patients with chronic hepatitis C than the 32 with chronic hepatitis B, including cryoglobulinemia (26% vs. 0%, $P < 0.001$), low CH_{50} levels (48% vs. 3%, $P = 0.012$), and the clonality of B cells (12% vs. 0%, $P < 0.01$). By multivariate analysis, HCV RNA in B cells was an independent factor associated with the presence of at least one marker for lymphoproliferation (odds ratio: 1.98 [95% confidence interval: 1.36–7.24], $P = 0.027$). Based on the results obtained, the infection of B cells with HCV would play an important role in the development of lymphoproliferative disorders. ***J. Med. Virol. 81:619–627, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: B cells; clonality; cryoglobulinemia; hepatitis C virus; lymphoproliferative disorders

INTRODUCTION

Hepatitis C virus (HCV) can induce chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [Tong et al., 1995; Ikeda et al., 1998]. In addition, some patients infected with HCV develop proliferative disorders of lymphocytes, such as mixed cryoglobulinemia [Agnello et al., 1992; Frangeul et al., 1996; Donada et al., 1998] and B-cell non-Hodgkin's lymphoma (NHL) [Ferri et al., 1994]. Cryoglobulinemia represents the oligoclonal proliferation of B cells and occurs in 19–56% of patients infected with HCV [Mazzaro et al., 1996; Donada et al., 1998; Weiner et al., 1998; Schmidt et al., 2000], while antibody to HCV (anti-HCV) and HCV RNA are detected more frequently in the patients with non-Hodgkin's lymphoma than in the general population (30% vs. 1.3%) [Ferri et al., 1994]. On the basis of these observations, cryoglobulinemia is considered to be a marker for lymphoproliferative disorders. In addition, rheumatoid factor (RF) in high titers and hypocomplementemia (low levels of C3, C4, or CH_{50}) are regarded as immunological markers for autoimmune disease and lymphoproliferation [Ramos-Casals et al., 2005]. In the patients with Sjögren's syndrome, for instance, hypocomplementemia was closely associated with the development of lymphoma [Ramos-Casals et al., 2005].

Although an epidemiological association has been noted between HCV infection and lymphoproliferative disorders, the pathogenic mechanisms underlying it have remained unclear. HCV would infect B cells persistently, and induce somatic mutations toward propagation in them. Recently, the replication of HCV was demonstrated in a B-cell line established from a patient infected with HCV [Sung et al., 2003], and

Grant sponsor: The Ministry of Education, Culture, Sports, Science and Technology of Japan (KAKENHI to T.I.); Grant number: 1859749; Grant sponsor: Grants-In-Aid for Research on Publicly Essential Drugs and Medical Devices (to T.I.); Grant number: KHC1018.

*Correspondence to: Takayoshi Ito, MD, Department of Gastroenterology, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan.
E-mail: tito@med.showa-u.ac.jp

Accepted 29 September 2008
DOI 10.1002/jmv.21388
Published online in Wiley InterScience (www.interscience.wiley.com)

somatic hypermutations in the immunoglobulin genes as well as proto-oncogenes were observed in a B-cell line infected with HCV [Machida et al., 2004]. These observations suggest direct and/or indirect effects of HCV infection of B cells on the induction of lymphoproliferative disorders. HCV infection by itself or stimulation by immune complexes containing viral antigens may trigger the clonal proliferation of B cells for the development of lymphoproliferative disorders in patients with chronic hepatitis C [Agnello, 1995; Ivanovski et al., 1998].

There is some evidence for the replication of HCV in peripheral blood mononuclear cells (PBMCs) of patients who are infected with HCV persistently [Moldvay et al., 1994; Lerat et al., 1998; Zignego and Brechot, 1999], although it is not known which of the T-, B-, and other cell-types supports the replication of HCV. It may replicate at very low levels in lymphoid cells, and they may serve as an extra-hepatic reservoir; this is implicated in recurrence and persistence of HCV infection in immunosuppressed individuals [Laskus et al., 2000]. Although current antiviral therapies can eliminate HCV from the serum, it may survive in lymphocytes thereafter [Pham et al., 2004; Radkowski et al., 2005]. The efficacy of interferon therapy on the HCV infection of lymphocytes is, as yet, uncertain.

The frequency of HCV infection of peripheral blood mononuclear cells (PBMCs) was examined in 75 patients with chronic hepatitis C, and HCV RNA levels were determined in $CD4^+$, $CD8^+$ T-cells, B-cells, and other cells. The replication of HCV in B cells was correlated subsequently with lymphoproliferative disorders.

MATERIALS AND METHODS

Patients

During 2003 through 2006, 75 patients infected with HCV were admitted to the Showa University Hospital, including two with acute hepatitis, 50 with chronic hepatitis, two with liver cirrhosis, 16 with hepatocellular carcinoma and five with non-Hodgkin's lymphoma. The diagnosis of HCV infection was confirmed in all these patients by the detection of anti-HCV and HCV RNA in the serum. All the patients were negative for hepatitis B surface antigen (HBsAg) or antibody to human immunodeficiency virus type-1. Serving as controls were 28 healthy individuals without HCV infection and 32 patients with chronic hepatitis B who did not have liver cirrhosis, hepatocellular carcinoma or non-Hodgkin's lymphoma. Informed consent was obtained from every participant for the purpose of this study, and the study was approved by the Ethics Committee of Showa University School of Medicine.

Isolation of Lymphoid Cells

PBMCs ($2.83 \pm 1.46 \times 10^7$) were obtained from whole blood (30 ml) by the centrifugation in a Ficoll/Hypaque gradient. Beads with the affinity for $CD8^+$ cells (MicroBeads®; Miltenyi Biotte, Bergisch Gladbach, Germany) were added to PBMCs, and the cell suspension was mixed well, incubated for 15 min at 4°C and centrifuged at 900*g* for 10 min in a tube. The tube was then placed on a magnet, and the supernatant floating free cells was transferred to another tube. The pellet containing $CD8^+$ cells was collected and stored at −80°C until use. $CD4^+$ and $CD19^+$ cells were separated from the supernatant using similar procedures. The remaining supernatant was pelleted to make the 'other cell' fraction. Each compartment of PBMCs contained $\sim 11 \times 10^5 - 10^6$ cells.

Quantitation of HCV RNA in Lymphoid Cells

HCV RNA was determined by reverse transcription-polymerase chain reaction (RT-PCR). Briefly, total RNA was extracted from each cellular compartment using Trizol® Reagent (Invitrogen, Carlsbad, CA). The RNA solution was stirred-up, and a portion (1 µl from the total of 50 µl) was reverse-transcribed by AMV® RT (Roche, Mannheim, Germany) and amplified by the single-step PCR for 40 cycles with appropriate primers (5′-CGC GCG ACT AGG AAG ACT TC-3′ and 5′-ATA GAG AAA GAG CAAC CA GG-3′) that are complementary to the 5′-UTR sequence. HCV RNA was determined in 100 ng of each RNA sample by the real-time RT-PCR using the primers described previously [Ito et al., 2001]; it has a detection range over 1.0–8.0 log copies. HCV RNA was recorded as positive for the samples with titers exceeding 1.0 log copies/100 ng, in order to exclude contamination of lymphoid cells with serum HCV RNA.

RT-PCR for Detecting Negative-Strand HCV RNA

Negative-strand HCV RNA was determined by the strand-specific RT-PCR. A portion of total RNA (1 µl from 50 µl) extracted from the B-cell fraction (10^5 cells) was reverse-transcribed with the sense primer (5′-AGA CAT CGG GCC AGA AGT GTC C-3′) complementary to a partial NS5B sequence of negative-strand HCV RNA, and amplified by the hot-started single-step RT-PCR for 40 cycles using the GeneAmp® EZ rTth RNA PCR kit (Applied Biosystems, Branchburg, CA) with the same primer and an antisense primer (5′-CGT TCA TCG GTT GGG GAG CAG G-3′) located downstream of it [Castillo et al., 2005]. Controls were negative- and positive-strand HCV RNA species that had been generated by in vitro transcription using the pCVJ4L6S plasmid [Yanagi et al., 1998]. Assays were performed in duplicate for each sample.

Serum Markers of Lymphoproliferative Disorders

Cryoglobulinemia was detected by a semi-quantitative centrifugation method. Briefly, blood samples were centrifuged at 600*g* for 20 min at 37°C. Sera were cooled to 4°C and left to stand for 48 h, and centrifuged again at 2,500*g* for 10 min at 4°C. The emergence of cryocrit at 4°C and its disappearance by warming up to 37°C for