liver ratio)が低い症例は，末梢血血小板減少症に対して脾摘を施行しても末梢血血小板数の回復が十分には得られなかったと報告している．末梢血血小板減少の要因として，肝臓への血小板集積は重要な病態である．

また今回の検討では対象の脾組織で，多くの症例に巨核球を認めた．巨核球にはTGF-β1の発現がみられ，巨核球を認める脾組織は巨核球を認めない脾組織よりTGF-β1の発現が高度であった．慢性肝炎において，TGF-β1は肝線維化に寄与する生理活性因子であることが報告されている[15)16)]．肝硬変では脾臓でのTGF-β1産生が増加し，脾臓で産生されたTGF-β1は門脈血を介して肝線維化に寄与すると報告されている[7)]．巨核球や血小板の内部には，さまざまな生理活性因子が包含されており，TGF-β1は巨核球や血小板に含まれる代表的な生理活性因子である[17)18)]．脾臓でのTGF-β1産生はマクロファージのかかわりが報告されているが，十分には明らかになっておらず，本研究の結果から脾臓でのTGF-β1産生に巨核球の関与も示唆された[7)8)]．

まとめ

肝臓への血小板集積が，肝硬変による末梢血血小板減少に対する脾摘の効果に関与するかを検討した．肝臓の壊死炎症反応が肝臓への血小板集積に寄与し，肝硬変による末梢血血小板減少に対する脾摘の効果を減弱させることが示唆された．肝脾組織内にみられる血小板，巨核球から，慢性肝炎に伴う末梢血血小板減少および肝線維化の治療につながる新しい見解を提案できる可能性があり，更なる検討が必要である．

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Original Article

Pathological characteristics of patients who develop hepatocellular carcinoma with negative results of both serous hepatitis B surface antigen and hepatitis C virus antibody

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Aim: We tried to characterize the pathological features of patients who developed hepatocellular carcinoma (HCC) with the negative results of both serous hepatitis B surface antigen and hepatitis C virus antibody (non-B, non-C).

Methods: In a multicenter study in Kyushu, Japan, we studied the histopathological characteristics of non-cancerous liver tissues in 129 patients (103 men and 26 women) with non-B, non-C HCC. The histological liver damage was evaluated for fibrosis (stage) and inflammation (grade) according to the Ludwig classification of chronic hepatitis. In addition, we examined the hepatitis B virus (HBV) genome in serum samples and liver tissues of 20 patients with non-B, non-C HCC.

Results: Positivity of serum hepatitis B core (HBc) antibody, alcohol abuse, diabetes and non-alcoholic steatohepatitis were present in 61 (47%), 76 (59%), 57 (44%) and eight (6%) patients, respectively. The degree of fibrosis was mild (stage 1.6 ± 1.2). The stage of patients with neither serum HBc antibody nor alcohol abuse was significantly lower than the stage of patients with HBc antibody and no alcohol abuse ($P < 0.05$). HBV genome was detected in 15 cancerous tissues (75%) and 16 non-cancerous liver tissues (80%) in 20 patients with non-B, non-C HCC. Only three of the 20 patients were positive for serum HBc antibody.

Conclusion: Non-B, non-C patients appear to develop HCC at a low stage of fibrosis. Occult hepatitis B virus infection is the major risk factor for HCC of non-B, non-C patients in Kyushu, Japan.

Key words: diabetes mellitus, hepatocellular carcinoma, large liver cell change, non-alcoholic steatohepatitis, non-B non-C, occult hepatitis B infection

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the fifth most common cancer worldwide.[1–3] HCC mostly occurs within an established background of chronic liver disease and cirrhosis. Although the risk factors for HCC, including infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), are well defined,[4–6] some patients with HCC in Japan have no confirmed chronic viral hepatitis, and the percentage of these patients is reportedly much higher in Western countries.[7–9]

Hepatocellular carcinoma cases without chronic viral hepatitis include patients who suffer from other chronic liver diseases predisposing to HCC, such as alcoholic liver disease,[9] hemochromatosis,[10] Budd–Chiari syndrome[11] and non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH).[12,13] In addition, there is a subpopulation of patients with HCC that develops from normal liver or liver tissue damaged from

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an unknown cause at a constant rate. The incidence and risk factors for HCC in patients without chronic viral hepatitis are not yet clear.

The Liver Cancer Study Group of Kyushu (LCSK) established a working group for the multicenter study to investigate the carcinogenesis of HCC without chronic viral hepatitis. The aim of the study described in the present paper was to clarify the pathological characteristics in non-cancerous liver tissues of patients who developed HCC with the negative results of both serous hepatitis B surface (HBs) antigen and HCV antibody (non-B, non-C) to investigate the carcinogenesis of HCC without chronic viral hepatitis.

METHODS

Tissues

WE STUDIED THE histopathological characteristics of 129 patients with non-B, non-C HCC between 1996 and 2006 with the LCSK and their affiliated hospital in the northern area of Kyushu, Japan. Patients with co-existing liver disease diagnosed by clinical and histological examination, such as autoimmune hepatitis, primary biliary cirrhosis, Budd–Chiari syndrome, hemochromatosis, Wilson disease and *Schistosomiasis japonica*, were excluded. Non-cancerous liver tissues were obtained by percutaneous biopsy or surgical operation (percutaneous biopsy, 65 patients; surgical operation, 64 patients).

In addition, we investigated HBV DNA in cryopreserved serum samples, HCC tissues and non-cancerous liver tissues that were obtained from 20 patients with non-B, non-C HCC in Kurume University Hospital in the period between 1997 and 2008.

This study was performed with informed consent obtained from patients for the use of their liver tissues and serum samples in the investigation and was approved by the ethical committee of Kurume University (approval ID no. 10004).

Histopathological examination of non-cancerous liver tissues

Each tissue was fixed with 10% formalin, embedded in paraffin, cut into 5-μm sections, and then used for histological analyses. The specimens were stained with hematoxylin–eosin and examined under a light microscope.

The histological liver damage of these specimens was evaluated for fibrosis and inflammation according to the Ludwig classification of chronic hepatitis.[14] The severity of fibrosis (stage of disease) was classified as none (stage 0), mild (portal fibrosis, stage 1), moderate (periportal fibrosis, stage 2), severe (bridging fibrosis with lobar distortion, stage 3) and cirrhosis (stage 4), and the inflammatory activity (grade of disease activity) was classified as none (grade 0), minimal (grade 1), mild (grade 2), moderate (grade 3) or severe (grade 4). In addition, the pathological features of hepatocytes, such as fatty change, ballooning and large liver cell change (LCC, Fig. 1), were evaluated.

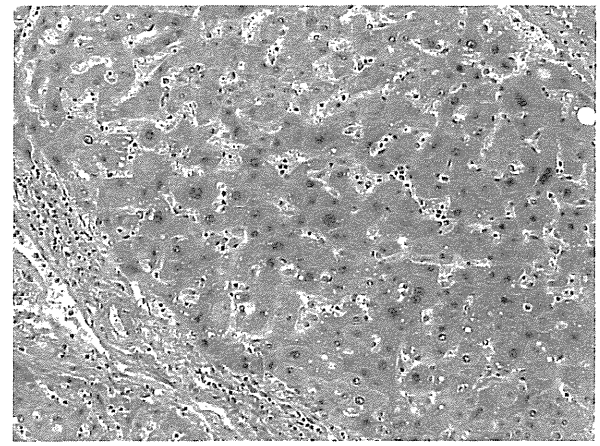

Figure 1 Microscopic findings (Large liver cell change). Large liver cell change is recognized as foci of cellular enlargement and nuclear pleomorphism, hyperchromasia and multinucleation (stained with hematoxylin and eosin, ×200).

Histopathological diagnosis and classification were performed by two pathologists (O. N. and M. K.).

Nucleic acid extraction from serum samples

Total nucleic acid was extracted from 300 μL of plasma using a commercially available kit (High Pure Viral Nucleic Acid Kit; Roche, Mannheim, Germany) according to the manufacturer's instructions. The extracted nucleic acid was eluted in 25 μL of elution buffer.

Nucleic acid extraction from liver tissues

The QIAamp DNA micro kit (QIAGEN, Hilden, Germany) was used to extract the nucleic acid from liver tissues (<10 mg) according to the manufacturer's instructions, with few changes. The DNA was eluted in 60 μL of AE buffer.

Quantification of HBV DNA (S, X, C)

Hepatitis B virus DNA was analyzed for the region of HBs, hepatitis B core (HBc), and hepatitis B x (HBx) by

Table 1 Nucleotide positions and sequences of TaqMan polymerase chain reaction primers and probes

	5′–3′	
S region		
Sense	TGTACAAAACCTTCGGACGGAAA	442–464
Antisense	TGCGAAAGCCCAGGATGATG	485–504
Probe	CTGCACTTGTATTCCC	465–480
Core region		
Sense	ACTGTGGTTTCACATTTCCTGTCTT	2072–2096
Antisense	GGCATTTGGTGGTCTGTAAGC	2163–2183
Probe	CCACACTCCAAAAGAC	2132–2147
X region		
Sense	CTACTGTTCAAGCCTCCAAGCT	1729–1750
Antisense	GCTCCAAATTCTTTATACGGGTCAATG	1778–1804
Probe	AAGCCACCCAAGGCAC	1751–1766

TaqMan real-time polymerase chain reaction (PCR) according to the manufacture's guidelines (Taqman Fast Universal PCR Master Mix; Applied Biosystems, Foster City, CA, USA). The oligonucleotide primers and probes that were optimized to adr of HBV subtype and specific for S, X and C region sequences, are summarized in Table 1. Plasmid pBRHBadr72 (full-length HBV DNA) was used as an internal standard in the quantitative real-time detection PCR. We used 8 μL nucleic acid from serum in our study for better sensitivity. The limit of sensitivity of our TaqMan real-time PCR methods ranged from 5 copies/well. The detection limit of our tests was 52 copies/mL. For quantification of HBV DNA from liver tissue, we used 3 μL nucleic acid, and a single copy housekeeping gene present in human, β-actin was assayed on the same sample. This was performed to estimate the number of cells presented in each PCR reaction. Serial dilutions of genomic DNA were used as standards to quantitate β-actin DNA from liver biopsies (TaqMan Beta-actin Detection Reagents; Applied Biosystems).

Enzymatic treatment with plasmid-safe adenosine triphosphate (ATP)-dependent DNase for detection of cccDNA

DNA extracted from liver tissue (<10 mg) was diluted to 60 μL in AE buffer. A 40-μL aliquot was put aside for detection of RC DNA (X, S, C regions) and β-actin DNA. The remaining 20 μL was digested with 25 units of Plasmid-Safe ATP-dependent DNase (Epicentre Technologies, Madison, WI, USA) for 30 min at 37°C in the presence of 10 × reaction buffer 5 μL, 25 mM ATP 2 μL, Plasmid-Safe DNase 1 μL and deionized distilled water 22 μL. Then, the reaction was inactivated by incubation at 70°C for 30 min. The mixture was purified using a QIAquick PCR Purification Kit (QIAGEN) and diluted by 40 μL EB buffer.

Quantification of HBV cccDNA

Hepatitis B virus cccDNA was tested using c-sel primers and probes as shown in Figure 2. We used 5 μL HBV cccDNA in the TaqMan real-time PCR. The internal standard was plasmid pBRHBadr72.

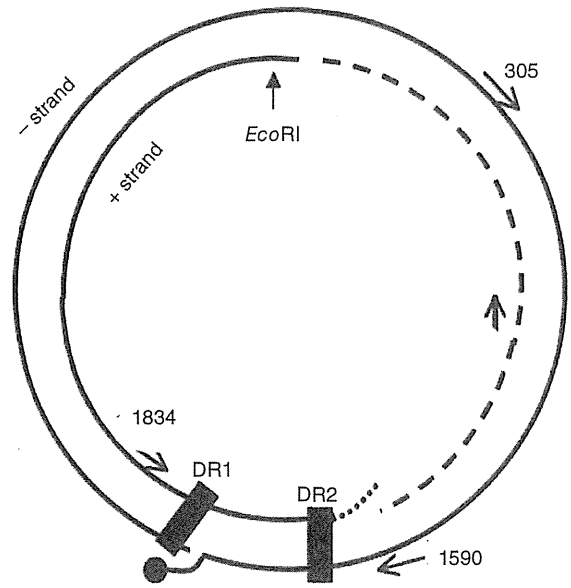

Figure 2 Quantification of HBV cccDNA. HBV cccDNA was tested using c-sel primers and probes. We used 5 μl HBV cccDNA in the Taqman real-time PCR. The internal standard was Plasmid pBRHBadr72.

Table 2 Clinical backgrounds of 129 patients examined by the LCSK

	AL (+)		AL (−)		Total
HBc (+)	40		21		61
	DM (+)	DM (−)	DM (+)	DM (−)	
	M: 15	M: 23	M: 4 (NASH: 1)	M: 8 (NASH: 1)	
	F: 1	F: 1	F: 3 (NASH: 1)	F: 6 (NASH: 1)	
	(A)	(B)	(C)	(D)	
HBc (−)	36		32		68
	DM (+)	DM (−)	DM (+)	DM (−)	
	M: 17	M: 18	M: 9	M: 9	
	F: 0	F: 1	F: 8 (NASH: 3)	F: 6 (NASH: 1)	
	(E)	(F)	(G)	(H)	
Total	76		53		129

(A) Stage 2.1 ± 1.4, grade 2.4 ± 1.2.
(B) Stage 1.8 ± 1.5, grade 1.9 ± 1.7.
(C) Stage 1.9 ± 1.1, grade 1.7 ± 1.4.
(D) Stage 1.8 ± 1.2, grade 2.2 ± 1.7.
(E) Stage 1.8 ± 1.1, grade 2.1 ± 1.2.
(F) Stage 1.3 ± 1.2, grade 1.9 ± 1.1.
(G) Stage 1.4 ± 1.3, grade 2.0 ± 1.4.
(H) Stage 1.0 ± 0.8, grade 1.4 ± 1.0.
Data are expressed as the mean ± standard deviation.
AL, alcohol abuse; DM, diabetes mellitus; F, female; HBc, serum HBc antibody; M, male; NASH, non-alcoholic steatohepatitis.

Statistical analysis

Arithmetic means and standard deviation (SD) of our data were calculated using a JMP software package (version 10.0; SAS Institute, Cary, NC, USA). All data are expressed as the mean ± SD, and *P*-values less than 5% were considered significant.

RESULTS

Clinical background

IN 129 PATIENTS with non-B, non-C HCC examined by the LCSK, there were 103 men and 26 women. The mean age of the men was 68 ± 10 years and the women 70 ± 11 years. Sixty-one patients (47%) were serum HBc antibody positive. Seventy-six patients (59%) abused alcohol (daily intake >60 g of ethanol for men, >40 g for women). Fifty-seven patients (44%) had diabetes mellitus. Most female patients (14 patients) had neither serum HBc antibody nor alcohol abuse (Table 2).

Clinical background and histological findings

Liver fibrosis and inflammation

Non-cancerous liver tissues of 129 patients with non-B, non-C HCC examined by the LCSK showed mild fibrosis and inflammation (grade 2.0 ± 1.4, stage 1.6 ± 1.2). The stage of patients with neither serum HBc antibody nor alcohol abuse was significantly lower than the stage of patients with HBc antibody and alcohol abuse (1.2 ± 1.1 vs 1.9 ± 1.4, $P < 0.03$), and the stage of patients with HBc antibody and no alcohol abuse (1.2 ± 1.1 vs 1.8 ± 1.1, $P < 0.05$) (Table 3). Patients with neither serum HBc antibody, alcohol abuse nor diabetes mellitus were of the lowest grade and stage in patients

Table 3 Clinical backgrounds and histological findings of 129 patients examined by the LCSK

	AL (+)	AL (−)	Total
HBc (+)	40	21	61
	Stage 1.9 ± 1.4	Stage 1.8 ± 1.1	
	Grade 2.1 ± 1.5	Grade 2.0 ± 1.6	
	**	*	
HBc (−)	36	32	68
	Stage 1.6 ± 1.2	Stage 1.2 ± 1.1	
	Grade 2.0 ± 1.6	Grade 1.7 ± 1.2	
Total	76	53	129

Data are expressed as the mean ± standard deviation.
*$P < 0.05$, **$P < 0.03$.
AL, alcohol abuse; HBc, serum HBc antibody; LCSK, Liver Cancer Study Group of Kyushu.

Table 4 Summary of histopathological findings in non-alcoholic patients examined by the LCSK

	Hepatic steatosis	Hepatocellular ballooning	Mallory–Denk body	Lipogranuloma	NASH
HBc (–), DM (–), $n = 15$	6	2	1	1	1
HBc (+), DM (–), $n = 14$	4	5	2	1	2
HBc (–), DM (+), $n = 17$	12	4	3	3	3
HBc (+), DM (+), $n = 7$	6	4	1	2	2

DM, diabetes mellitus; HBc, serum HBc antibody; LCSK, Liver Cancer Study Group of Kyushu; NASH, non-alcoholic steatohepatitis.

examined by the LCSK (stage 1.0 ± 0.8, grade 1.4 ± 1.0; Table 2).

Pathological features of hepatocytes

Non-cancerous liver tissues of 129 patients with non-B, non-C HCC examined by the LCSK showed various pathological features. In 53 patients without alcohol abuse, eight patients were identified as having NASH. There were six female patients of the eight patients with NASH (Table 2). NAFLD was present in 28 patients of the 53 non-alcoholic patients. Of 28 patients with NAFLD, diabetes mellitus was present in 18 (64%) patients (Table 4). LCC was observed in 52 of the 61 patients (85%) with serum HBc antibody, and 44 of the 68 patients (65%) without serum HBc antibody (Fig. 3).

HBV DNA in serum samples and liver tissues

In cryopreserved serum samples, HCC tissues and non-cancerous liver tissues obtained from 20 patients with non-B, non-C HCC, HBV DNA was detected in one serum sample (5%), 15 HCC tissues (75%) and 16 non-cancerous liver tissues (80%) (Table 5). In only two patients, both HCC tissues and non-cancerous liver tissues were negative for HBV DNA detection. In addition, only three of the 20 patients were positive for serum HBc antibody.

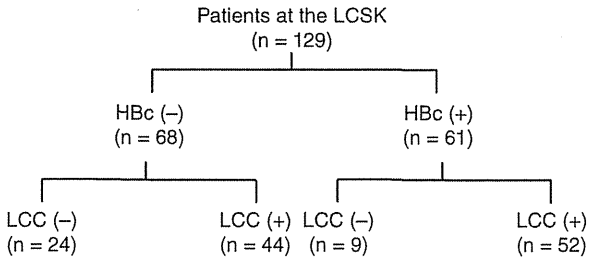

Figure 3 Large liver cell change of 129 patients examined by the Liver Cancer Study Group of Kyushu. There are 96 patients (74%) with large liver cell change (LCC) in 129 patients with non-B, non-C hepatocellular carcinoma. Large liver cell change is observed in 52 of the 61 patients (85%) with serum hepatitis B core (HBc) antibody, and 44/68 patients (65%) without serum HBc antibody.

DISCUSSION

RECENTLY, THE AVAILABILITY of vaccines for HBV has decreased the proportion of patients with HBs antigen by preventing mother-to-infant infection.[15] Antiviral therapy in patients with HCV may prevent carcinogenesis.[16,17] The incidence of HCC associated with HBV or HCV is thus forecast to decrease in Japan, whereas HCC without hepatitis virus infection will remain.[7,18] In this study, we demonstrated the pathological characteristics in non-cancerous liver tissues of patients with non-B, non-C HCC.

Patients with non-B, non-C HCC have various clinical backgrounds, such as serum HBc antibody, alcohol abuse and diabetes mellitus. Abe *et al.*[8] reported 64 patients with non-B, non-C HCC in Tokyo, Japan. Positivity of serum HBc antibody, alcohol abuse and diabetes mellitus was present in 34 (53.1%), 46 (71.9%) and 29 (45.3%) patients, respectively. Yano *et al.*[19] reported 22 patients with non-B, non-C HCC in Saga, Kyushu, Japan. Positivity of serum HBc antibody was present in 16 (72.7%) patients. In this study, we studied 129 patients with non-B, non-C HCC in the northern area of Kyushu, Japan. Positivity of serum HBc antibody, alcohol abuse and diabetes mellitus was present in 61 (47%), 76 (59%) and 57 (44%) patients, respectively. We considered that there were regional differences with respect to the frequency of the non-B, non-C HCC development risk factors, such as positivity of serum HBc antibody, alcohol abuse and diabetes mellitus.

Hepatitis C virus infection, HBV infection, alcohol abuse and NASH are the main causes of liver fibrosis.[20] In this study, non-cancerous liver tissues of patients with neither serum HBc antibody, alcohol abuse nor

Table 5 Summary of HBV DNA in serum samples and liver tissues

Case no.	Serum samples		Liver tissues							
	HBc Ab	Surface	HCC tissues				Non-cancerous tissues			
			Surface	X	Core	cccDNA	Surface	X	Core	cccDNA
1	−	−	−	+	+	−	−	+	−	−
2	+	−	−	+	−	−	−	+	−	−
3	+	−	+	−	−	−	+	+	+	+
4	−	−	−	−	−	−	−	−	−	−
5	−	−	+	−	−	−	+	+	+	−
6	−	−	−	+	−	−	+	+	−	−
7	−	−	−	−	−	−	−	−	+	−
8	−	+	−	+	−	−	−	−	−	−
9	−	−	−	−	−	−	+	−	−	−
10	−	−	+	+	+	−	+	+	+	+
11	−	−	−	+	−	−	+	+	+	−
12	−	−	+	+	+	−	+	+	−	−
13	−	−	+	+	−	−	+	+	−	−
14	−	−	+	+	−	−	+	−	−	−
15	+	−	+	−	−	+	+	+	+	−
16	−	−	+	+	+	−	−	−	+	−
17	−	−	−	−	−	−	−	−	−	−
18	−	−	−	−	−	−	+	+	−	−
19	−	−	+	+	+	−	−	+	−	−
20	−	−	−	+	−	−	−	−	−	−

Ab, antibody; core, HBV C region; HBc, hepatitis B core; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; surface, HBV S region; X, HBV X region.

diabetes mellitus showed a slight degree of fibrosis. Positivity of serum HBc, alcohol abuse and diabetes mellitus may be an additive factor for liver fibrosis.

In patients with chronic viral hepatitis, HCC mostly occurs within liver cirrhosis. Liver cirrhosis results in liver failure, portal hypertension and increased risk of carcinogenesis.[21] In this study, non-cancerous liver tissues of 129 patients with non-B, non-C HCC examined by the LCSK showed mild fibrosis and inflammation. We consider that patients without chronic viral hepatitis may have another risk factor for HCC apart from liver fibrosis.

It has been suggested that NASH may account for a substantial portion of cryptogenic HCC cases.[22] In this study, 129 patients with non-B, non-C HCC examined by the LCSK included 28 patients with NAFLD. Of these, diabetes mellitus was present in 18 (64%) patients. NAFLD has been widely accepted as a possible etiological factor in the development of non-B, non-C HCC; because of the fact that diabetes mellitus was common in patients with NAFLD, insulin resistance may further facilitate the development of HCC.

In addition, there were 96 patients (74%) with LCC of the 129 patients with non-B, non-C HCC examined by the LCSK in this study. LCC was observed in 52 of the 61 patients (85%) with serum HBc antibody, and 44 of the 68 patients (65%) without serum HBc antibody. LCC is characterized by individually scattered or clusters of hepatocytes with atypia, measuring less than 1 mm in diameter, which do not form circumscribed nodules, and have been often found in chronic liver disease.[23,24] LCC is recognized under the microscope as the foci of cellular enlargement and nuclear pleomorphism, hyperchromasia and multinucleation. Although LCC is frequently found in various liver diseases and easily recognized even under low-power magnification due to the characteristic cytological features, its pathological significance is still under debate. LCC has been reported to be observed in various liver diseases such as autoimmune hepatitis, alcoholic cirrhosis and cholestatic liver, although it is more prevalent in HBV-related chronic liver disease.[24–26] It was reported that LCC in HBV-related chronic liver disease demonstrated molecular characteristics different from that in chronic

cholestasis.[27] We also confirmed in this study that HBV DNA was detected in 15 HCC tissues (75%) and 16 non-cancerous liver tissues (80%) obtained from 20 patients with non-B, non-C HCC, although only three of the 20 patients were positive for serum HBc antibody. Based on these findings, we consider that occult HBV infection may be present in a considerable number of patients without serum HBc antibody and occult HBV infection is the major risk factor for the development of HCC in patients without chronic viral hepatitis in the northern area of Kyushu, Japan. Recently, a relationship between the incidence of non-B, non-C HCC and occult HBV infection has been reported.[28–30] The HBV genome persists in a high percentage of HBs antigen negative patients, with and without antibodies against the virus, who develop HCC.[28] It was reported that HBV DNA was detectable in a high proportion of HCC patients without HBs antigen in Japan.[29,30]

In conclusion, the results obtained in the present study indicate that non-cancerous liver tissues of patients with non-B, non-C HCC showed mild fibrosis and inflammation, although serum HBc antibody, alcohol abuse and diabetes mellitus may be additional factors for liver fibrosis. In addition, HBV DNA was detectable in a high proportion of patients with non-B, non-C HCC. Further study of the interaction between HBV DNA in liver tissues and cellular protein will contribute to understanding the involvement of HBV in the HCC of unknown etiology.

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Original Article

Influence of splenectomy in patients with liver cirrhosis and hypersplenism

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Aim: Splenectomy improves hypersplenic thrombocytopenia in cirrhotic patients with hypersplenism. However, the long-term influence of splenectomy has not been clarified. We examined whether splenectomy improved liver fibrosis and caused immunological changes.

Methods: We collected liver and spleen specimens and peripheral blood (PB) from 26 patients with hepatitis C virus-related liver cirrhosis. An immunohistochemical examination of CD4, CD8, forkhead box P3, granzyme B and transforming growth factor-β1, and Masson-trichrome stain were performed in spleen and liver tissues and in seven cases of follow-up liver biopsy sections obtained after splenectomy. We obtained PB before and at various intervals after splenectomy. We also examined the ratio of $CD4^+$ and $CD8^+$ lymphocytes in PB using flow cytometry.

Results: We observed improvements in liver fibrosis in four biopsy specimens obtained after splenectomy, in which fibrotic areas significantly decreased from 19.5% to 8.2% ($P < 0.05$). Increases were also observed in the ratio of $CD8^+$ cells in PB after splenectomy, which resulted in a significant decrease in the $CD4^+/CD8^+$ ratio ($P < 0.001$). The carcinogenic rate in patients with a $CD4^+ : CD8^+$ ratio that decreased by more than 0.5 at 1 month after splenectomy was significantly lower than that in patients with a ratio that decreased by less than 0.5 ($P < 0.05$).

Conclusion: Splenectomy may improve liver fibrosis and cause beneficial immunological changes in cirrhotic patients with hepatitis. Improvements in antitumor mechanisms can be also expected.

Key words: $CD4^+$ cytotoxic T lymphocytes, $CD8^+$ cytotoxic T lymphocytes, liver cirrhosis, liver fibrosis, splenectomy

INTRODUCTION

SPLENECTOMY IS A common treatment used to improve hypersplenic thrombocytopenia in cirrhotic patients with splenomegaly in Japan.[1–7] Splenectomy has recently been applied as another option to cure hepatocellular carcinoma (HCC) and for cirrhotic patients with no potential donor for liver transplantation. Thus, the clinical application of splenectomy has been expanded; however, the immunophysiology of the spleen in cirrhotic patients and the long-term outcome after splenectomy have not been clarified.[8–14] This study was designed to clarify the long-term changes and prediction of HCC development following splenectomy, with a focus on hepatic fibrosis and immunology. Regarding hepatic fibrosis, Akahoshi *et al.* reported that transforming growth factor (TGF)-β1 derived from the spleen could have an inhibitory role in healing liver cirrhosis by inhibiting the regeneration of the damaged liver[15] and we experimentally confirmed that splenectomy significantly reduced liver fibrosis and decreased TGF-β1 in the serum of a dimethylnitrosamine-induced cirrhotic rat model.[16] However, no studies have yet described a reduction in hepatic fibrosis following splenectomy in humans.

The spleen plays an important role in the immune response; however, the functional aspects of the spleen in cirrhotic patients with hepatitis C virus (HCV) infection are largely unknown.[2,17] Hashimoto *et al.* reported that splenectomy was followed by an increased ratio of interferon (IFN)-γ to interleukin (IL)-10 and a reduction in programmed death (PD)-1-expressing $CD4^+$ T cells in peripheral blood (PB).[7] In order to clarify chronological changes in immunity after splenectomy, we examined liver and spleen tissues and sera to assess $CD4^+$ and

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$CD8^+$ cytotoxic T lymphocytes (CTL) and regulatory T (Treg) cells.[18,19] TGF-β1 was also examined as it is a multifunctional cytokine that inhibits the growth of tumor cells[20–23] and liver regeneration by facilitating tissue fibrosis in the liver.[16]

Host immunoreactions against cancer were shown to be closely related to cellular immunity by $CD8^+$ CTL and Treg cells, produced by T lymphocytes, and $CD8^+$ CTL in particular.[19] The level of Treg cells, characterized by the expression of forkhead box P3 (FOXP3) transcription factor in the PB and tumor tissues of patients with HCC, was elevated and appeared to be negatively correlated with prognosis.[21,24,25]

In the present study, we examined whether splenectomy could improve liver fibrosis, cause immunological changes, especially in CTL, or be used to predict the risk of carcinogenesis.

METHODS

Patients and samples (Table 1)

AT THE DEPARTMENT of Surgery, Kurume University Hospital, 26 patients (Child A, 16 cases; Child B/C, 10 cases) with HCV-related liver cirrhosis (with HCC, seven cases; without HCC, 19 cases) and hypersplenism underwent splenectomy (splenectomy group). The purpose of splenectomy was to improve hypersplenic thrombocytopenia and introduce IFN for clearance of the HCV virus. Forty-eight patients who underwent hepatectomy due to liver tumors were recruited as controls (control group 1). PB samples from 10 healthy adult volunteers (control group 2) and spleen tissues obtained by splenectomy from seven patients because of trauma (control group 3) were also used as controls. In addition, all patients were HIV negative. Patients received no medical treatment except splenectomy during the study period. All samples were studied after obtaining the appropriate institutional informed consent. We also obtained permission from the ethical review board.

Liver tissue

A total of 26 pieces from the resected liver specimens of patients with HCV-related liver cirrhosis and hypersplenism who underwent splenectomy were also examined for the immunohistochemical expression of $CD4^+$

Table 1 Subject characteristics

Variables	Results
Splenectomy group: splenectomy (26 cases, seven with HCC, 19 without HCC)	
Age, median (range)	60.4 ± 1.36 (46–75)
Sex (male/female)	12/14
Virus infection (HCV^+)	26
Fibrosis (F0/F1/F2/F3/F4)	0/0/0/0/26
Child–Pugh classification (A/B/C)	16/8/2
Tumor nodules (presence/absence)	7/19
Weight of the spleen (g)	510.4 ± 55.6 (125–1065)
Control 1: hepatectomy with HCC (48 cases)	
Age, median (range)	70.5 ± 1.33 (42–82)
Sex, male/female	29/19
Virus infection (HCV^+)	40
Fibrosis (F0/F1/F2/F3/F4)	8/10/10/10/10
Tumor nodules (presence/absence)	48/0
Control 2: healthy adult volunteers (10 cases)	
Age, median (range)	40.1 ± 2.97 (32–57)
Sex (male/female)	3/7
Control 3: splenectomy control (seven cases; trauma)	
Age, median (range)	59.8 ± 6.27 (36–82)
Sex (male/female)	6/1

Continuous variables are expressed as the mean ± standard deviation.
Fibrosis: F0, no fibrosis in the portal tract; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, numerous septa without cirrhosis; F4, cirrhosis.
HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

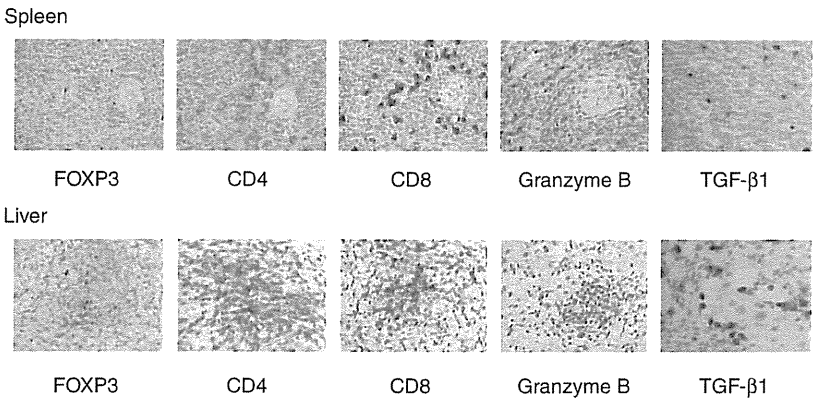

Figure 1 Immunohistochemical staining of spleen and liver specimens with forkhead box P3 (FOXP3), CD4, CD8, granzyme B and transforming growth factor (TGF)-β1 in the spleen and liver.

lymphocytes, $CD8^+$ lymphocytes, FOXP3, granzyme B and TGF-β1 positive cells (Fig. 1). We classified liver specimens into five stages according to the degree of fibrosis as follows: F0, no fibrosis in the portal tract; F1, portal fibrosis without septa; F2, portal fibrosis with a few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis. We collected resected liver specimens from 10 cases each of F1, F2, F3 and F4 with HCV-related liver disease. We also collected specimens from eight cases of liver hemangioma of F0 with both negative hepatitis B surface antigen and HCV antibody. Follow-up liver biopsy sections were obtained from the same part of the liver if possible from seven of the 26 patients at various intervals after splenectomy (Table 2). These sections were used for CD4 and CD8 immunostaining and Masson-trichrome staining for the morphometric evaluation of fibrotic areas.

Spleen tissue

A total of 26 spleens with HCV-related liver cirrhosis and hypersplenism were examined for the immunohistochemical expression of CD4 positive lymphocytes, CD8 positive lymphocytes, FOXP3, granzyme B and TGF-β1 positive cells. We measured the same parameters in spleens from the seven control cases in control group 3 as a non-cirrhotic control (Fig. 1). Spleen and liver tissues were pathologically assessed by two pathologists (Y. N. and M. K.).

Peripheral blood cells

Peripheral blood samples were serially collected from 26 patients with HCV-related liver cirrhosis and hypersplenism just before and 14 days, 1 month, 3 months, 6 months and 1 year after splenectomy. We examined the ratio of $CD4^+$ T cells to all lymphocytes, $CD8^+$ T cells to all lymphocytes, and the $CD4^+/CD8^+$ ratio in PB samples using flow cytometry. TGF-β1 levels in PB were also measured using enzyme-linked immunoassays in the sera just before and 14 days, 1 month, 3 months, 6 months and 1 year after splenectomy. Patients were excluded from the protocol if IFN or other therapeutics were introduced for the liver disease. Ten healthy adult

Table 2 Clinical and pathological findings of 7 patients who underwent follow-up liver biopsies

Case	Age	Sex	Activity	Child–Pugh (score)	CD4/8	Follow-up range (days)	Before (%)	After (%)	Rate of change
1	63	M	1	A (5)	1.73	581	6.59	18.31	2.78
2	58	M	2	A (5)	1.22	24	7.38	8.99	1.22
3	58	M	2	B (7)	1.57	333	9.92	12.02	1.21
4	52	M	2	A (5)	1.08	431	16.71	5.10	0.30
5	74	M	2	A (6)	0.63	353	20.02	6.31	0.32
6	53	F	2	A (6)	0.93	248	30.03	13.34	0.44
7	59	M	2	A (5)	0.95	42	11.27	8.05	0.71

Activity: A0, none; A1, portal inflammation only; A2, mild interface hepatitis; A3, moderate interface hepatitis; A4, severe interface hepatitis.
Before, the rate of fibrotic areas before splenectomy; after, the rate of fibrotic areas after splenectomy.

volunteers in control group 2 without a history of liver disease or splenomegaly were also recruited as controls, and samples were collected only once.

Immunohistochemical analysis

All fresh specimens were fixed by 10% formalin, and paraffin-embedded tissue samples were cut at a thickness of 4 μm, examined on a coated slide glass, and labeled with the following antibodies using the Bond-Max autostainer (Leica Microsystems, Newcastle, UK) and DAKO autostainer (DakoCytomation, Glostrup, Denmark): CD4 (×200; Leica Microsystems), CD8 (×200; Leica Microsystems), granzyme B (×50; Leica Microsystems), TGF-β1 (×300; Santa Cruz Biotechnology, Heidelberg, Germany) and FOXP3 (×600; Abcam, Cambridge, MA, USA).

Immunohistochemical examinations with CD4, CD8, granzyme B and TGF-β1 were performed on the same fully automated Bond-Max system using onboard heat-induced antigen retrieval with ER2 for 10 min and the Refine polymer detection system (Leica Microsystems). 3,3′-Diaminobenzidine-tetrachloride (DAB) was used as the chromogen for all immunostaining. FOXP3 immunostaining was carried out using the DAKO autostainer with the ChemMate ENVISION method (DakoCytomation). Briefly, specimens were boiled in a microwave for 30 min in 1 mmol/L ethylenediaminetetraacetic acid, pH 9.0, and target retrieval solution (DakoCytomation) to recover antigens, and the specimens were then incubated with the antibody at 4°C overnight. After washing in Tris-buffered saline (TBS), slides were incubated with the labeled polymer-horseradish peroxidase secondary antibody for 30 min at room temperature. After washing in TBS, slides were visualized using DAB.

Detection of immune function using flow cytometry

T-lymphocyte subsets in PB such as CD4, CD8 and CD4/8 were determined by flow cytometry, and the monoclonal antibodies of CD4 and CD8 (labeled CD4-FITC, CD-8-RD1) were purchased from Beckman Coulter (Danvers, MA, USA).

Result assessment

For assessment criteria for lymphocytes and other positive cell counts, the number of lymphocytes and other positive cells were counted in 20 areas within a specimen under high-power fields (×40 objective, ×10 eyepiece). Ten areas of white and red pulp were assessed in the spleen, and 10 periportal areas and 10 hepatic lobule areas (Fig. 1) were assessed in a non-tumor area of the liver.

Morphometric analysis (computer image analysis) was performed in the following manner on specimens stained with Masson-trichrome. The equipment used to assess morphometry consisted of a light microscope, a three-color charge-coupled device camera, and a high resolution computer image analysis system (WinRooF software package version 6.1; Mitani, Fukui, Japan). The magnified images (×40) of specimens captured by the camera mounted on the microscope were sent to the image analyzing computer. Collagen fibers stained with Masson-trichrome were then selected. In this study, this scanning procedure was repeated 10 times in random areas. The area of fibrosis (AF) was defined as the ratio (%) of the whole area of collagen fibers to that of the liver tissue scanned.

Statistical analyses

Statistical analysis was performed using Student's *t*-test. A *P*-value of less than 0.05 was considered to be significant.

The follow-up time was calculated as the interval between the date of surgery and intervention of the medical treatment, last follow up or recognition of HCC. Survival rates or failure rates were analyzed with the Kaplan–Meier method using the log-rank test to assess differences between curves. A *P*-value of less than 0.05 was considered to be significant. Statistical calculations were performed using the JMP software package (release 10, SAS Institute, Cary, NC, USA).

RESULTS

Liver

IN THE SEVEN follow-up liver biopsy sections (Table 2) available for histological examination, liver fibrosis in the hepatic lobules improved from F4 to F3 in four cases (cases 4–7: average, 268.5 ± 168.6 days; range, 42–431 days) (Fig. 2a). Improvements were not observed in the remaining three cases (cases 1–3: average, 312 ± 279.1 days; range, 24–581 days) (Fig. 2b). There were no statistical differences in the duration between the improvement cases and non-improvement cases ($P = 0.80$). Conducting an evaluation was difficult because only a few specimens were available; however, no significant differences in clinical profiles were observed among the seven patients. In four of these cases (cases 4–7), the ratio significantly

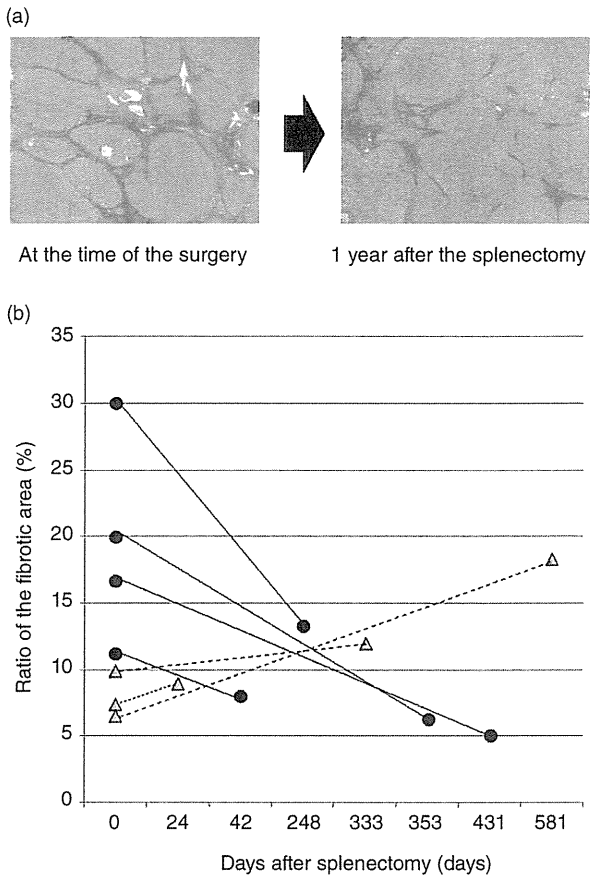

Figure 2 (a) Improvements in liver fibrosis. Distortions in hepatic lobules improved in the liver biopsy sections of four cases after splenectomy, and fibrotic areas significantly decreased from 19.5% to 8.2% in these sections. (b) Changes in the fibrotic areas of seven patients at various intervals. •—• shows patients in whom the fibrotic area significantly decreased after splenectomy. △···△ shows patients in whom fibrosis deteriorated.

decreased from 19.5% to 8.2% ($P < 0.05$) (Fig. 2b), while the average AF in the remaining three cases (cases 1–3) increased from 8.0% to 13.1% ($P = 0.15$). The four cases of improved fibrosis were all Child–Pugh A, and one of the three cases that showed no improvement was Child–Pugh B. In addition, AF before splenectomy was slightly higher in the improvement cases than in the non-improvement cases, while the $CD4^+/CD8^+$ ratio before splenectomy was lower in the improvement cases than in the non-improvement cases ($P < 0.05$). Histopathologically, $CD4^+$ and $CD8^+$ lymphocytes were mainly seen in the periportal area, and $CD4^+$ lymphocytes were rarely seen in the hepatic lobules. The epithelial cells, fibroblasts, monocytes and macrophages also produced TGF-β1.[4,21,26] However, we picked up and counted the TGF-β1 positive cells that were seen in the lymphocytes and found that these cells were distributed diffusely in the hepatic lobules and periportal area. The distribution pattern of Treg and granzyme B was the same as that of $CD4^+$ and $CD8^+$ lymphocytes, respectively. No significant differences were observed in the $CD4^+/CD8^+$ ratio ($P = 0.21$) in liver specimens, regardless of the association of HCC. The $CD4^+/CD8^+$ ratio ($P < 0.05$) and $FOXP3/CD4^+$ ratio ($P < 0.001$) significantly increased with the progression of liver fibrosis (from F0 to F4). However, the granzyme $B/CD8^+$ ratio was approximately constant, and was unrelated to the progression of liver fibrosis ($P = 0.32$).

The number of TGF-β1 positive cells in livers with HCC was slightly higher than that in livers without ($P = 0.06$), and the number of TGF-β1 positive cells also significantly increased with the progression of liver fibrosis ($P < 0.001$) (Fig. 3).

Spleen

Histopathologically, $CD4^+$ and $CD8^+$ lymphocytes were found more in the white pulp than in the red pulp. The results of the clinicopathological analysis showed that the $CD4^+/CD8^+$ ratio in spleens with HCV-related liver cirrhosis and hypersplenism was higher than that in the spleens of control group 3 ($P = 0.06$). The $FOXP3/CD4^+$ ratio in control group 3 was higher than that in cases of hypersplenism ($P < 0.05$), and no significant differences

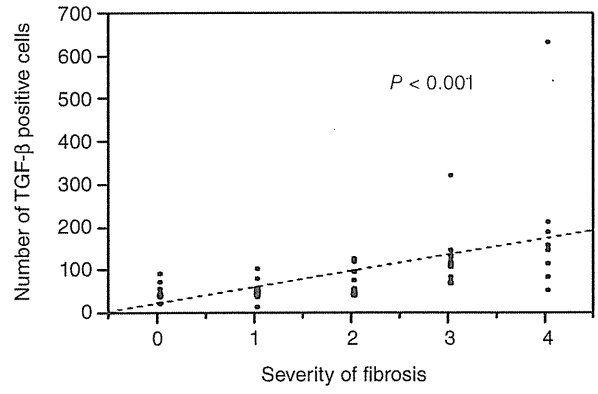

Figure 3 Correlation between transforming growth factor (TGF)-β1 positive cells and fibrosis in the liver. The number of TGF-β1 positive cells also significantly increased with the progression of liver fibrosis.

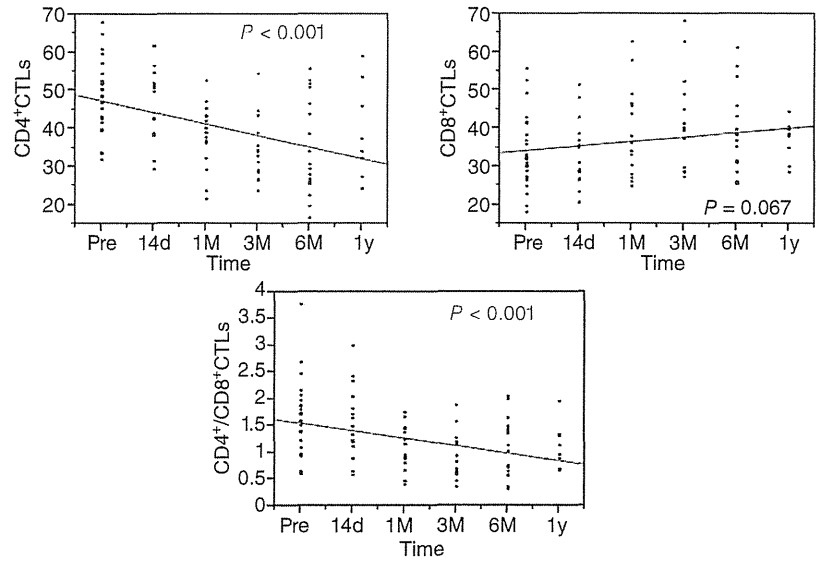

Figure 4 Changes in peripheral blood after splenectomy. pre, preoperative; d, days; M, months; y, year. The ratio of $CD4^+$ T cells to all lymphocytes significantly decreased 1 year after splenectomy, while the ratio of $CD8^+$ T cells to all lymphocytes slightly increased, resulting in a significant decrease in the $CD4^+/CD8^+$ ratio.

in the granzyme B/$CD8^+$ ratio ($P = 0.82$) were observed between the splenectomy group and control group 3 (data not shown).

Peripheral blood

The ratio of $CD4^+$ T cells to all lymphocytes and the $CD4^+/CD8^+$ ratio in PB samples obtained from 26 patients before splenectomy were significantly higher than those from control group 2 ($P < 0.01$, $P < 0.05$). In contrast, the ratio of $CD4^+$ T cells to all lymphocytes significantly decreased 1 year after splenectomy ($P < 0.001$), while the ratio of $CD8^+$ T cells to all lymphocytes slightly increased ($P = 0.07$), resulting in a significant decrease in the $CD4^+/CD8^+$ ratio ($P < 0.001$) (Fig. 4).

Transforming growth factor-β levels were higher in PB samples from patients with HCC than in those without. TGF-β1 levels slightly increased in PB samples 1 month after splenectomy, then decreased, and subsequently returned to the level measured before splenectomy in 1 year.

Relationship of the $CD4^+/CD8^+$ ratio between PB and the spleen or liver

In the splenectomy group, the $CD4^+/CD8^+$ ratio in PB had a significant positive correlation with the $CD4^+/CD8^+$ ratio in the spleen ($P < 0.05$), and was also positively associated with the liver ($P = 0.07$). As a result, a significant positive correlation was observed between the $CD4^+/CD8^+$ ratio in the spleen and that in the liver ($P < 0.05$) (Fig. 5).

Correlation between the $CD4^+/CD8^+$ ratio and clinical prognosis

We compared the $CD4^+/CD8^+$ ratio between PB obtained pre-splenectomy and 1 month after splenectomy ($n = 19$). The median of differences between pre-splenectomy and 1 month after splenectomy was 0.5. The occurrence of HCC was significantly lower in cases in which the difference in the $CD4^+/CD8^+$ ratio between the perioperative period and 1 month later was over 0.5 (≥0.5 vs <0.5, $P < 0.05$) (Fig. 6a).

A positive correlation in PB was observed between the $CD4^+/CD8^+$ ratio before splenectomy and differences in the $CD4^+/CD8^+$ ratio between pre-splenectomy and 1 month after splenectomy ($P < 0.001$). As the median of the preoperative $CD4^+/CD8^+$ ratio was 1.7, the postoperative (1 month after splenectomy) $CD4^+/CD8^+$ ratio significantly decreased in groups in which the preoperative value was larger than 1.7 (Fig. 6b,c).

DISCUSSION

PREVIOUS STUDIES HAVE shown that splenectomy was effective in improving pancytopenia, the decompression of portal hyperpressure and liver function.[1,2,27,28] Morinaga *et al.* reported that splenectomy

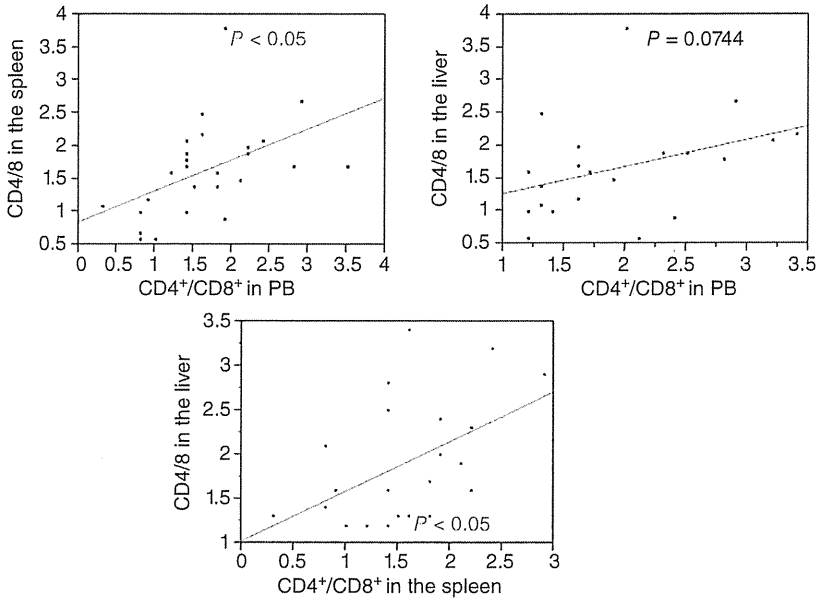

Figure 5 Correlations between the $CD4^+/CD8^+$ ratios in the spleen, liver and peripheral blood (PB). A significant positive correlation was observed between the $CD4^+/CD8^+$ ratio in the spleen and that in the liver.

significantly improved liver fibrosis with a reduction in plasma TGF-β1 levels in the rat. However, all these reports of hepatic fibrosis were conducted in animal models[1,16,29,30] whereas the present study described improvements in liver fibrosis after splenectomy in humans. Interestingly, the $CD4^+/CD8^+$ ratio changed after splenectomy without other treatment. However, many confounding factors may be implicated in this change. It is likely that patients with a high fibrotic area in their liver specimens had a high $CD4^+/CD8^+$ ratio;

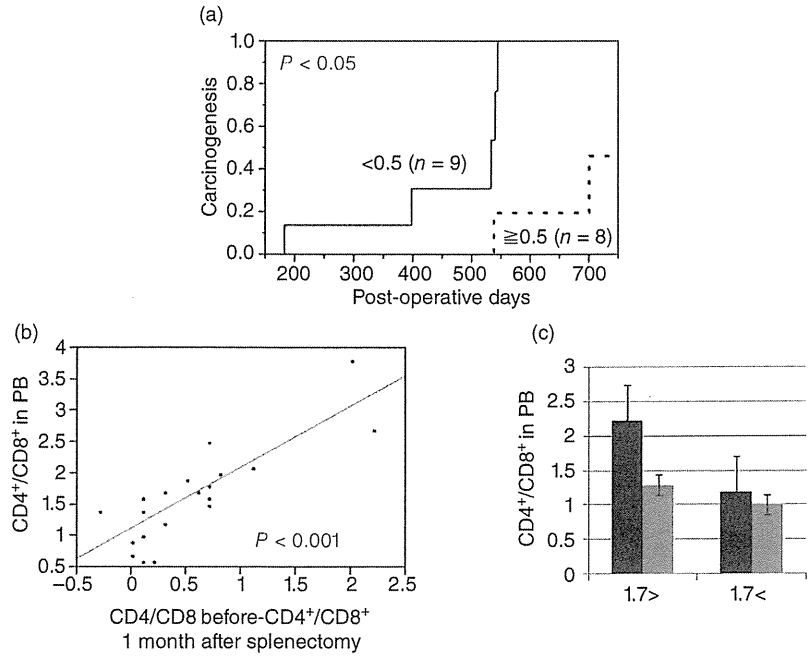

Figure 6 (a) Correlation between carcinogenesis, the perioperative period and 1 month later. The occurrence of hepatocellular carcinoma was significantly lower in cases in which the difference in the $CD4^+/CD8^+$ ratio between the perioperative period and 1 month later was over 0.5. (b,c) Correlation in peripheral blood (PB) between the $CD4^+/CD8^+$ ratio before surgery and differences in the $CD4^+/CD8^+$ ratios before splenectomy and 1 month after splenectomy. (b) A positive correlation in PB was observed between the $CD4^+/CD8^+$ ratio before splenectomy and differences in the $CD4^+/CD8^+$ ratio between pre-splenectomy and 1 month after splenectomy. (c) The postoperative (1 month after splenectomy) $CD4^+/CD8^+$ ratio significantly decreased in groups in which the preoperative value was larger than 1.7. ■, pre; ■, post.

therefore, we may expect a decrease in the $CD4^+/CD8^+$ ratio after splenectomy. A decrease in Treg cells that stimulate TGF-β1 may lead to alleviation of fibrosis.

Because the immune function of $CD4^+$ CTL, $CD8^+$ CTL and the $CD4^+/CD8^+$ ratio is affected by a wide variety of factors including recent exercise, poor nutrition and coincident acute viral infections, it is difficult to evaluate immune function using only $CD4^+$ CTL, $CD8^+$ CTL and the $CD4^+/CD8^+$ ratio. However, in our study, the ratio of $CD4^+$ T cells to all lymphocytes in PB was significantly decreased in cirrhotic patients after splenectomy, while the ratio of $CD8^+$ T cells to all lymphocytes slightly increased, resulting in a significant decrease in the $CD4^+/CD8^+$ ratio. The $CD4^+/CD8^+$ ratios in PB, spleens and livers were significantly higher in patients with hypersplenism and in those in whom liver fibrosis had progressed than in the controls. As a positive correlation was observed between the $CD4^+/CD8^+$ ratios in the spleens, livers and PB, it is possible to expect to predict the immunological state of the liver and spleen from the immunological state of PB. In addition, carcinogenesis was significantly lower in groups in which a large difference in the $CD4^+/CD8^+$ ratio was observed between before and after splenectomy or in those with a high $CD4^+/CD8^+$ ratio before splenectomy though there were few cases that we could observe. The $CD4^+/CD8^+$ ratio is likely to be a key parameter for appropriate tumor-infiltrating lymphocyte function, and was shown to be different in different types of cancer.[2,31–35] Host immune responses to cancer were reported to depend on T lymphocytes, particularly $CD8^+$ lymphocytes.[18,19,24,36–39] An increase in their ratio after splenectomy and the consequent decrease in the $CD4^+/CD8^+$ ratio observed in this study may be a positive change in terms of immunology against HCC. Such a change was particularly marked in patients with a high $CD4^+/CD8^+$ ratio before splenectomy.

In our study, the $CD4^+/CD8^+$ ratio also significantly increased as the fibrosis of non-tumor areas in the liver tissue progressed. These significant differences were observed regardless of the HCC status. Although the cause of these differences is unknown, it appears to depend on the background of histological factors in the liver such as fibrosis. Many studies have investigated the relationship between tumors, Treg and TGF-β.[20–22,25,40] Guo-He *et al.* showed that the expression of TGF-β appeared to be positively correlated with Treg in HCC tissue. The 5-year survival rate was significantly lower in patients with HCC tissues with high Treg cell infiltration than in those with low infiltration.[20,22,36,41] Our study also revealed that Treg cells were positively correlated with TGF-β1 positive cells even in "non-tumor areas" of liver tissue, and that TGF-β1 positive cells were positively correlated with liver fibrosis. There were no significant differences of TGF-β1 before and after splenectomy. The reason for the chronological changes in TGF-β1 levels after splenectomy is unknown because various factors including platelets may be involved in the production of TGF-β1. We also found a slightly higher number of TGF-β1 positive cells in non-tumor areas in the liver tissue of patients with HCC than in those without. Furthermore, the number of TGF-β1 positive cells significantly increased with the progression of liver fibrosis.[4,21,26,42]

In conclusion, splenectomy in cirrhotic patients with hepatitis may be able to improve liver fibrosis, cause beneficial immunological changes and lower the risk of carcinogenesis. It seems necessary to accumulate further cases to establish a convincing conclusion.

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