Suppression of malignant metastasis is considered to be partially dependent on the angiogenic phenotype of the primary tumor.[25,26] That is, in the clinical setting, development of multiple metastases sometimes occurs immediately after surgical resection of a primary tumor. This phenomenon has been explained by humoral factors derived from the primary tumor. Removal of the primary tumor may lead to a decrease in circulatory antiangiogenic factors produced by the primary tumor and to progression of metastasis.[27–29] Thus, humoral systemic antiangiogenic agents may be involved in both metastasis and tumor progression. However, chronic liver diseases, especially associated with viral hepatitis B or C, are clearly recognized as premalignant conditions for HCC.[15] In this regard, we investigated the serum PEDF protein concentration in patients with CH, LC, and HCC. In our clinical analysis, PEDF was constitutively and abundantly detected in the sera of healthy volunteers, and PEDF protein concentration was found to be decreased in the sera to levels proportionate with the progression of premalignant liver diseases. However, evaluation of serum samples of patients with compensated LC (Child-Pugh classification A or B; n = 5) and decompensated LC (Child-Pugh classification C; n = 5) showed no differences in serum PEDF concentration between the two groups ($P = 0.293$, data not shown). The number of samples may be too small or PEDF expression may be fully suppressed even in compensated LC before progression to decompensated LC. Next, we evaluated the PEDF mRNA by Northern blot analysis in several liver tissues to confirm the direct causal relationship between PEDF expression and liver disease. There was a tendency for PEDF mRNA expression to decrease to levels proportionate with the progression of liver disease. However, the number of samples that we could obtain from liver surgery in this study was not sufficient for proper statistical analysis (n = 2 for each group). Thus, in this study, we could not provide reliable data on PEDF mRNA expression in liver tissues of patients with various liver diseases. However, previous studies reported a liver-specific high expression of PEDF compared with other organs.[13,14] Hence, serum PEDF concentrations seem to be dependent on hepatic protein production capacity. In this regard, virus protein, hepatic fibrosis, or inflammation itself may affect PEDF production from the liver. Further research is required to clarify the mechanism of the suppressive effect of PEDF in chronic liver disease. In this study, because PEDF protein concentrations in LC and HCC complicating LC were approximately equivalent, development of HCC apparently did not influence the serum PEDF protein concentration. However, reduction of serum PEDF concentration may alter tissue surrounding HCC toward angiogenic conditions and may contribute to the progression of HCC. In other words, PEDF may function as a tumor suppresser in some HCC patients or cell lines. Further investigation, in a larger number of patients with liver diseases or other malignant diseases, may be needed because the sample number of this clinical study was quite small. Moreover, the only other report of circulating PEDF reported 5 μg/mL of this protein in plasma by Western blot analysis using known amounts of purified PEDF.[30] In this study, we used a commercial ELISA kit to measure serum PEDF concentrations. The difference in the two studies may be caused by differences in the method used for measurement, sample type, or both. In this regard, a previous study used the same ELISA kit and reported that PEDF concentration in human vitreous fluid was 1 to 9 ng/mL, although other investigators who used their own ELISA system reported the level to be at 1 to 2.5 μg/mL in the same fluid.[31]

In this study, the CM derived from all PEDF-overexpressing HCC cell lines efficiently inhibited proliferation and migration of HUVECs regardless of the level of PEDF expression, whereas the three HCC cell lines expressed massive levels of VEGF in the CM (data not shown). In addition, overexpression of PEDF in preestablished subcutaneous HCC tumors in nude mice resulted in efficient suppression of tumor progression. When PEDF expression decreased because of the limited period of plasmid expression system, the tumor started to escape growth suppression. Moreover, PEDF protein induced from pcDNA3-PEDF-transfected tumors was not detected in the mouse serum (data not shown). These results indicate that the local concentration of antiangiogenic factors is important for tumor growth inhibition. Indeed, the therapeutic limitation of systemic administration of antiangiogenic compounds has been reported.[32,33] In this regard, antiangiogenic gene therapy may be an attractive strategy, because gene induction may increase the local concentration of the protein product from the therapeutic gene in the tumor. Indeed, we have already reported the enhanced growth inhibition of angiostatin gene-induced PLC/PRF/5.[34] Recently, Wang et al.[23] reported the antitumor effects of systemic or intratumoral administration of adenovirus encoding PEDF in a mouse HCC and lung carcinoma model. However, this model is confounded because the viral vector, including adenovirus, could induce a critical adverse reaction.[35] Although nonviral gene delivery systems are less efficient at inducing transgene expression and have shorter-term expression (compared with viral delivery systems), as shown in the present study, adverse reactions are thought to be less frequent. In the present study, we used a plasmid vector encoding PEDF. Despite the expected low efficiency of gene induction,

marked growth inhibition of preestablished tumors was demonstrated. Therefore, the present study indicates that a sufficient bystander effect was achieved by this strategy, and if the transgene is expressed intratumorally, highly efficient therapeutic gene induction is not necessarily required. HCCs seem to be very sensitive to vascular starvation.

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□ CASE REPORT □

Nonalcoholic Steatohepatitis with Improved Hepatic Fibrosis after Weight Reduction

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Abstract

A 65-year-old woman was admitted to our hospital for an investigation of liver dysfunction. She had mild obesity with hyperlipidemia, but no history of alcohol abuse. Other known causes of liver dysfunction, such as viruses, autoimmunity and drug effects, were excluded. The liver histology was consistent with nonalcoholic steatohepatitis (NASH). After diagnosis of NASH, the patient started diet and exercise therapy and, in parallel with weight reduction, her liver function improved. One year after the therapy, a liver biopsy showed that steatosis, necroinflammation and even fibrosis were improved. Hence, here we report a case of NASH in which weight reduction was effective in improving both biochemical and histological findings.

(Internal Medicine 43: 289–294, 2004)

Key words: nonalcoholic steatohepatitis, weight reduction, hepatic fibrosis

Introduction

Obesity is an epidemic that is currently recognized as a major public health problem worldwide. In recent years, the number of obese patients has also increased in Japan. Obesity is a risk factor for various diseases such as type II diabetes, hyperlipidemia, hypertension and cardiovascular disease. Furthermore, obesity is a condition that is often reported in association with nonalcoholic fatty liver diseases, including nonalcoholic steatohepatitis (NASH) (1–4).

NASH is a condition characterized by a histologic picture similar to alcoholic liver injury, but without the presence of alcohol abuse (1–4). Many patients with NASH have underlying risk factors such as obesity, diabetes mellitus and hyperlipidemia (1–5). The natural history and the long-term prognosis of NASH are not well understood, but the available data suggest that NASH is a benign disease in most patients. However, it was reported that 43% of patients with NASH had histologic progression and in approximately 8% to 17% of patients with NASH it can lead to cirrhosis with related complications (6–8).

There are a few previous reports on the effect of diet and exercise therapy on the clinical features of NASH (9–12). However, the effect of weight reduction on histologic findings, and especially on fibrosis, is not fully understood. Several drug therapies for NASH have been reported to be potentially useful, but the number of patients in these studies was small. Here, we describe a patient with NASH who was treated using diet and exercise therapy, and consequently showed both biochemical and histologic improvement of the liver.

Case Report

The patient was a 65-year-old woman who had suffered from liver dysfunction from 1998. She had no history of blood transfusion, or alcohol or drug abuse. Although she had received injections of 60 ml of stronger neo-minophagen C (SNMC) three times a week, her elevated transaminase levels were sustained. She was admitted to our hospital on May 9, 2001 for an examination of her liver dysfunction. A physical examination on admission showed mild obesity (body mass index; BMI 25.1 kg/m^2). No hypertension or hepatomegaly were noted. The laboratory data on admission are shown in Table 1.

Blood biochemistry tests showed an aspartate aminotransferase (AST) level of 210 IU/*l*, alanine aminotransferase (ALT) 231 IU/*l*, lactate dehydrogenase (LDH) 293 IU/*l* and alkaline phosphatase (ALP) 385 IU/*l*. The fasting blood glu-

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Table 1. Laboratory Data on Admission

Peripheral blood		Blood Chemistry			
White blood cells	4,900/mm³	Total protein	8.1 g/dl	Iron	125 μg/dl
Neutorophil	57%	Albumin	5.1 g/dl	Ferritin	225.3 ng/dl
Eosinophil	3%	Total bilirubin	1.2 mg/dl	Copper	101 μg/dl
Basophil	0%	Asparate aminotransferase	210 IU/l	Ceruloplasmin	22.4 mg/dl
Lymphocyte	29%	Alanin aminotransferase	231 IU/l	Serology	
Monocyte	11%	Alkaline phosphatase	385 IU/l	Immunoglobulin G	1150 mg/dl
Red blood cells	474×10⁴/mm³	Lactate dehydrogenase	293 IU/l	Immunoglobulin A	64 mg/dl
Hemoglobin	14.8 g/dl	γ-glutamyltranspeptidase	68 IU/l	Immunoglobulin M	642 mg/dl
Hematocrit	42.50%	Blood urea nitrogen	15 mg/dl	HBsAg	0.1 COI
Platelet	16.0×10⁴/mm³	Creatinine	0.7 mg/dl	HBc-Ab	96.2%
Coagulation		Na	145 mEq/l	HBcAb (×200)	16.1%
Prothrombin time	94%	K	4.1 mEq/l	Anti-HCV antibody	(–)
APTT	29.9 sec.	Cl	109 mEq/l	HBV DNA	(–)
		Total cholesterol	231 mg/dl	HEV RNA	(–)
		Triglyceride	155 mg/dl	TTV DNA	(–)
		Blood glucose	86 mg/dl	Antinuclear antibody	(–)
		HbA1c	4.7%	Antimitochondral antibody	(–)
		Fasting insulin	13.2 μU/ml	Antismooth muscle antibody	(–)
		HOMA	2.8		
		ACE	25.9 IU/l		

ATT: activated partial thromboplastine time, HOMA: homeostasis model of assessment, ACE: angiotensin converting enzyme, HBsAg: hepatitis B surface antigen, HBc-Ab: anti-hepatitis B core antibody, HCV: hepatitis C virus, COI: cut off index, HBV: hepatitis B virus, HEV: hepatitis E virus, TTV: TT virus, DNA: deoxyribonucleic acid, RNA: ribonucleic acid.

cose, hemoglobin A1c and fasting insulin levels were all normal. The patient's homeostasis model of assessment (HOMA) score (13), which is commonly used as a surrogate marker for insulin resistance, was elevated to 2.80. Total cholesterol and triglyceride levels were elevated to 231 mg/dl and 155 mg/dl, respectively. The patient tested negative for various viral markers, including hepatitis B surface antigen (HBs-Ag), anti-hepatitis B core antibody (HBc-Ab), antibody to hepatitis C virus (anti-HCV), HBV DNA, TT virus (TTV) DNA and hepatitis E virus (HEV) RNA. HBsAg, and HBc-Ab were assayed using commercially available radioimmunoassay kits (Dainabot, Tokyo, Japan). Anti-HCV was determined using a third-generation enzyme-linked immunoadsorbent assay (Ortho Diagnostics Systems, Tokyo, Japan). Serum HBV DNA was tested by using polymerase chain reaction (PCR) reported previously (14), TTV DNA was tested by using PCR kit (Institute of Immunology Co., Ltd. Tokyo, Japan) and HEV RNA was tested by RT-PCR (Mitsubishi Kagaku Bio-Clinical Laboratories, Inc. Tokyo, Japan). Autoantibodies, including anti-nuclear antibody (ANA), anti-mitochondrial antibody (AMA), and anti-smooth muscle antibody (ASMA) were also negative. The autoimmune hepatitis (AIH) score by the revised international criteria (15) for diagnosis of AIH was nine, which was not considered to be AIH. Serum iron, copper, ceruloplasmin and angiotensin-converting enzyme level were normal. Fibrosing markers, hyaluronic acid and procollagen III peptide (P-III-P) were elevated (hyaluronic acid 186 ng/ml, normal range ≤50 ng/ml; P-III-P 1.4 U/ml, normal range ≤1.0 U/ml). An ultrasonography examination suggested that the echogenicity of the liver was diffusely increased, compared to that of the kidneys. A liver biopsy was performed on the fourth hospital day to evaluate liver histology. A specimen evaluated using light microscopy showed the presence of macrovesicular and microvesicular steatosis, spotty necrosis, mild to moderate inflammatory cell infiltration and moderate perivenular, perisinusoidal and portal fibrosis (Fig. 1) with one focus of porto-portal bridging fibrosis [Grade 2 and Stage 3, as categorized by Brunt et al. (16)]. Based on these findings, the patient was diagnosed with nonalcoholic steatohepatitis (NASH).

After diagnosis of NASH, diet therapy (1,280 kcal/day; 25 kcal/kg/day) and exercise therapy (180 kcal/day) was started. The patient made a continuous effort toward achieving weight reduction. Consequently, her body weight decreased from 58 kg to 53 kg (BMI 22.9 kg/m²) and her elevated level of transaminases normalized after 12 months of diet and exercise therapy, in parallel with her weight reduction (Fig. 2). However, the patient's HOMA score did not decrease (HOMA 3.0).

On July 15, 2002, the patient was re-admitted to our hospital for examination of her liver and a second liver biopsy was performed. Compared with the initial biopsy, histologic findings such as steatosis, necroinflammation and even fibrosis of the liver, were improved (Fig. 3: Grade 1 and Stage 2). Based on laboratory data, hyaluronic acid and P-III-P had normalized to 28.2 ng/ml and 0.9 U/ml, respectively.

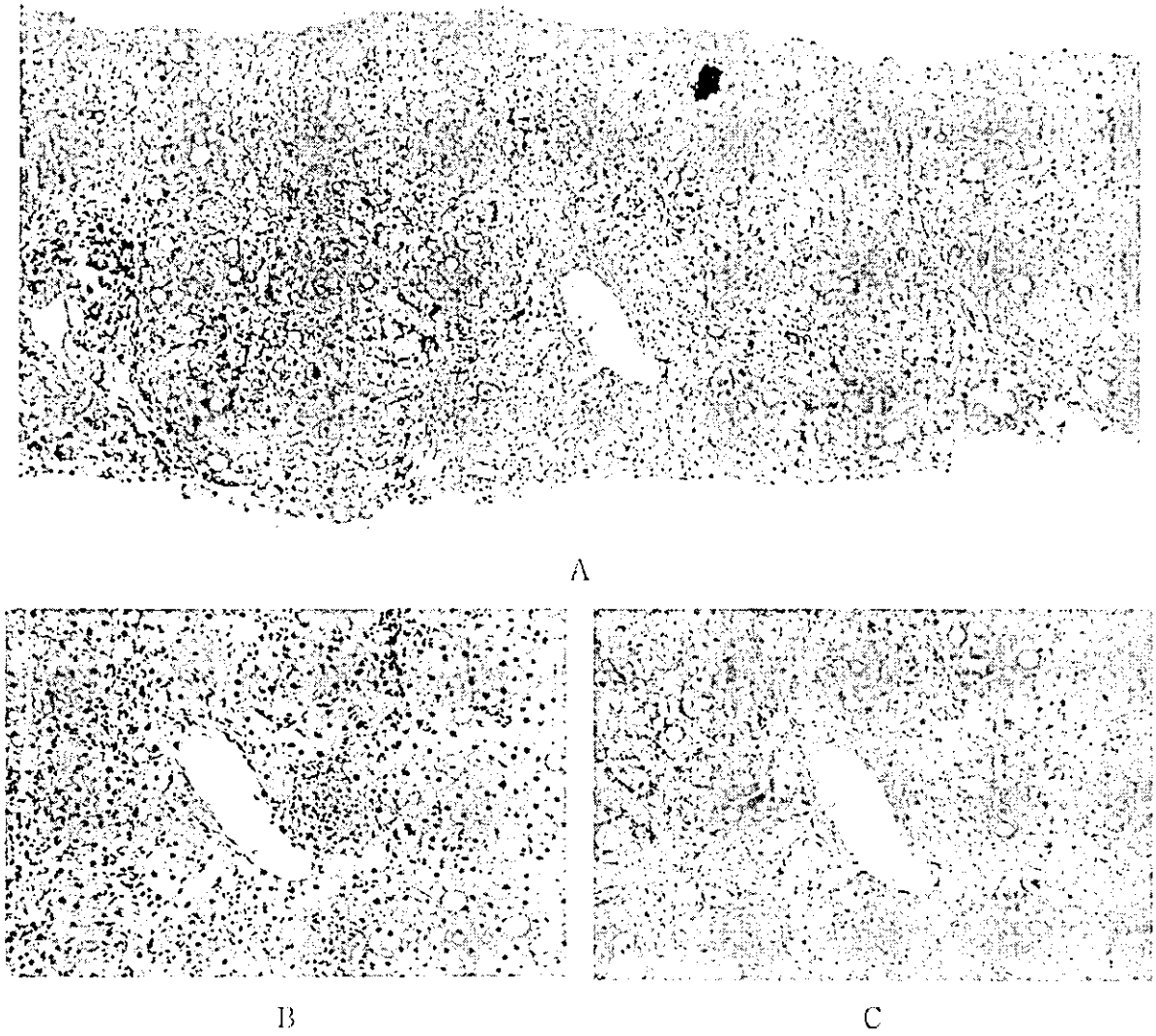

Figure 1. Liver biopsy specimens before diet and exercise therapy. A. Moderate macrovesicular and microvesicular steatosis, and moderate fibrosis in the centrilobular and portal areas (Azan-Mallory, ×40). B. Moderate steatosis, spotty necrosis with lymphocyte and polymorphonuclear leukocyte infiltration and ballooned hepatocytes around the central vein (hematoxylin and eosin, ×100). C. Extensive perivenular and perisinusoidal fibrosis in the centrilobular (zone 3) area (Azan-Mallory, ×100).

Discussion

NASH is defined histologically when a combination of macrovesicular steatosis, hepatocyte injury and necrosis, mixed inflammatory cell infiltration and variable degrees of fibrosis are observed in the absence of chronic abuse of alcohol (1–4). The histologic findings for the patient described above were consistent with NASH, showing 30 to 50% macrovesicular and microvesicular steatosis, porto-portal bridging fibrosis, piecemeal necrosis, and mild-to-moderate inflammatory cell infiltration. NASH is mainly associated with obesity and diabetes mellitus, hypercholesterolemia and hypertriglyceridemia (1–5), and the patient also had some of these risk factors.

Although in most cases fatty liver disease does not progress to more severe liver diseases, approximately 20 to 30% of patients have histologic signs of fibrosis and necroinflammation, indicating the presence of NASH. Furthermore, some cases of NASH are at a higher risk of developing cirrhosis, terminal liver failure, and hepatocellular carcinoma (7, 8, 17, 18). It has been reported that obese persons of relatively advanced age (≥45 years), and those with diabetes mellitus, a greater degree of hepatic steatosis, and higher grades of hepatic inflammation have a risk for progression to

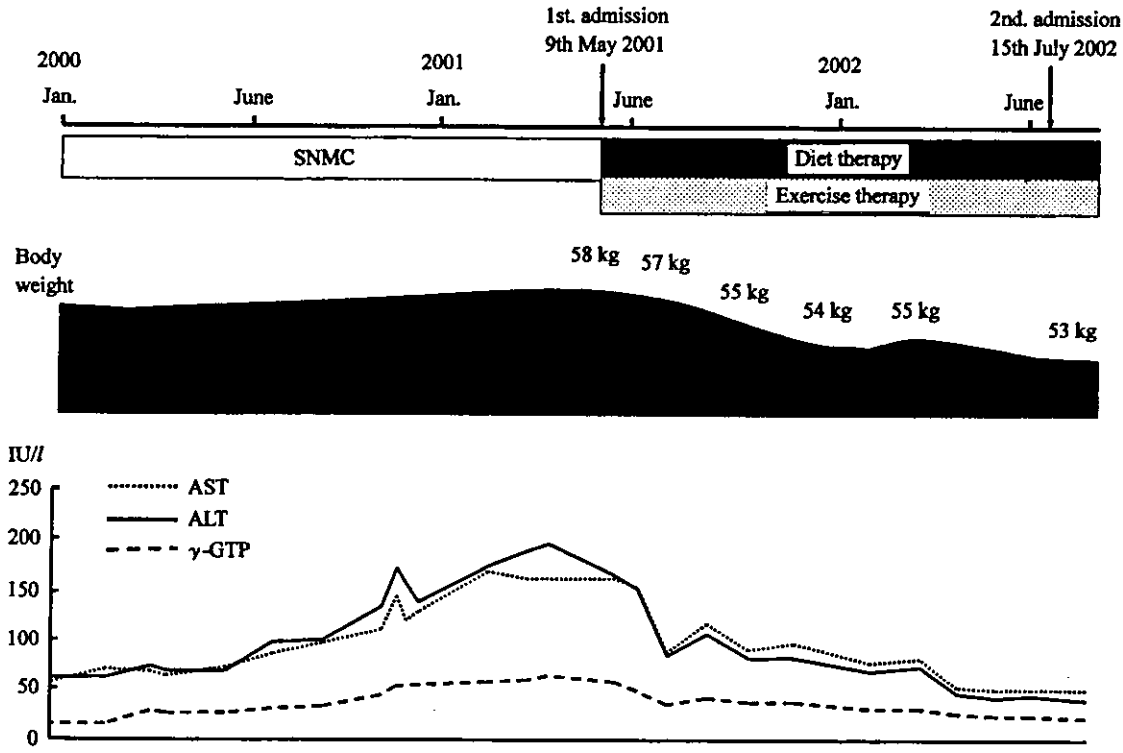

Figure 2. The clinical course of the patient. In parallel with weight reduction, liver dysfunction improved during diet and exercise therapy. AST: aspartate aminotransferase, ALT: alanine aminotransferase, γ-GTP: γ-glutamyltransferase, P-III-P: procollagen III peptide.

cirrhosis (19).

Current treatments for NASH are unproven. Improvement of liver chemistry, but variable changes in histology, have been reported after weight reduction in a small number of patients with NASH (9–11). The rate of weight reduction is important and may play a critical role in determining whether liver histologic findings improve or worsen. Rapid weight reduction has been associated with exacerbation of steatohepatitis in obese patients, and histologic exacerbation has been observed when the rate of weight reduction exceeded 1,600 g per week (20). Hence, weight reduction should be moderate and should also be monitored carefully. However, the most effective rate of weight reduction still has to be established. In the current case, the patient achieved 5 kg weight reduction during one year of diet and exercise therapy. Originally elevated transaminase levels decreased in parallel with this weight reduction. Furthermore, an improvement in liver histology, including the grade of steatosis, inflammation and the stage of fibrosis, were observed after weight reduction. These findings indicate that weight reduction is a useful therapy for NASH, but large prospective studies are needed to confirm this suggestion.

The mechanisms by which weight reduction improves hepatic inflammation and fibrosis in NASH patients remain unclear. The pathogenesis of NASH is multifactorial. In a model for the development of NASH (21), it is suggested that insulin resistance is an important factor in the accumulation of hepatocellular fat. Other factors, such as genetic mutations, excess carbohydrates, drugs and toxins may also contribute to hepatic steatosis. An excess of fat in the liver predisposes some individuals to hepatocellular injury, caused by the direct cellular toxicity of excess free fatty acids, oxidative stress and lipid peroxidation, or other mechanisms. Although, it is commonly known that diet and exercise therapy alter insulin sensitivity, the current patient showed no significant change in insulin resistance during therapy. These findings might suggest that hepatic steatosis does not depend on insulin resistance only.

Drugs such as gemfibrozil (22), ursodeoxycholic acid (23), vitamin E (α-tocopherol) (24) and metformin (25) have been shown to be promising treatments for NASH. However, studies of these drugs have been limited to only small numbers of patients, and they have also had variation in the definition of NASH and insufficient evaluation of treatment outcomes. In addition, medical therapies for NASH have had other problems, such as costs and side effects. While the es-

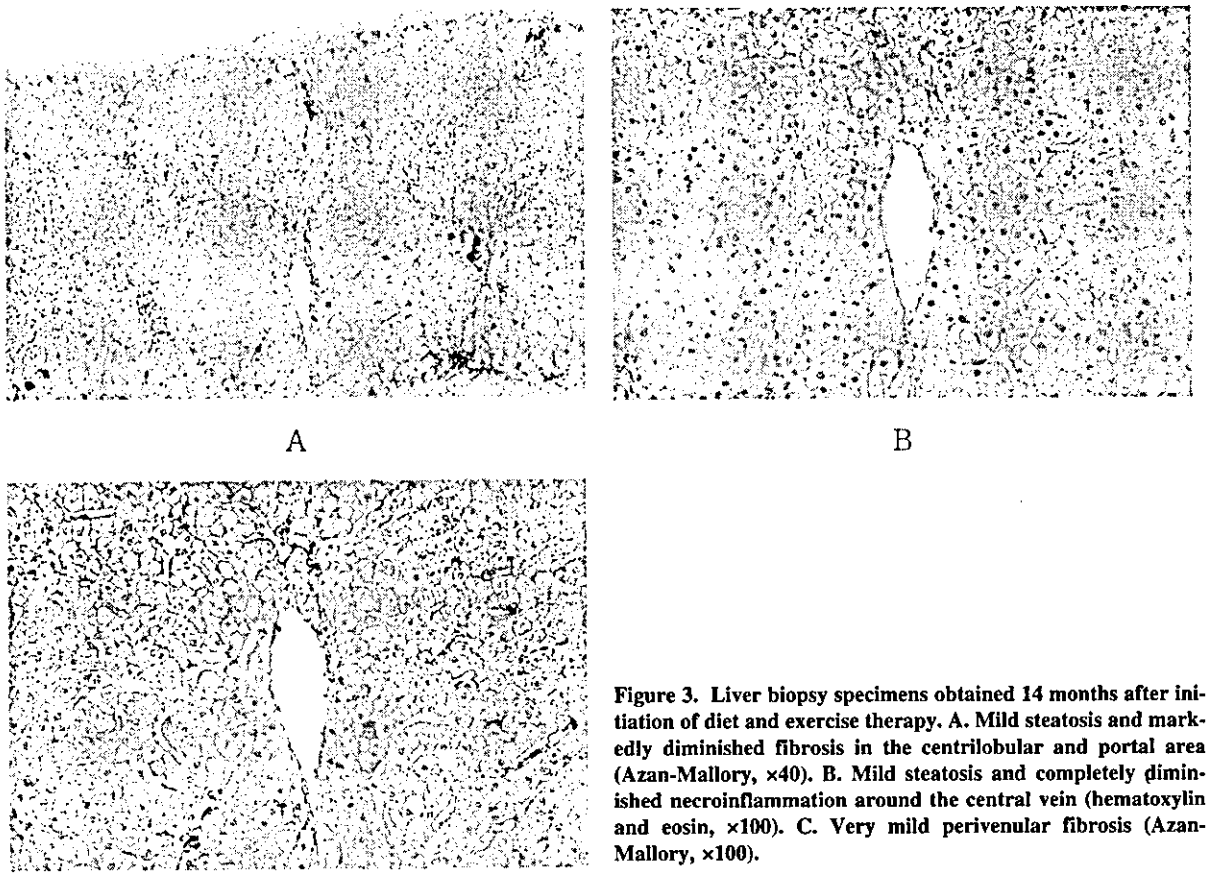

Figure 3. Liver biopsy specimens obtained 14 months after initiation of diet and exercise therapy. A. Mild steatosis and markedly diminished fibrosis in the centrilobular and portal area (Azan-Mallory, ×40). B. Mild steatosis and completely diminished necroinflammation around the central vein (hematoxylin and eosin, ×100). C. Very mild perivenular fibrosis (Azan-Mallory, ×100).

tablishment of an effective therapeutic approach is awaited, we believe that gradual weight reduction might be a useful first step in NASH therapy.

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HEPATOLOGY

High viral load is a risk factor for hepatocellular carcinoma in patients with chronic hepatitis B virus infection

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Abstract

Background and Aims: Hepatitis B virus (HBV) is considered a major risk factor for the progression to liver cirrhosis and hepatocellular carcinoma (HCC). The serum level of HBV-DNA is correlated with progression of the disease. The aim of the present study was to determine the relationship between the level of HBV-DNA and hepatocarcinogenesis in patients with chronic HBV infection.

Methods: The authors studied 73 patients who were diagnosed with chronic HBV infection at Nagasaki University Hospital (Nagasaki, Japan) between January 1980 and December 1999. The significance of age, sex, habitual drinking, serum alanine aminotransferase level, HBV viral load, interferon treatment, hepatic fibrosis and hepatic inflammation on the development of HCC were examined using univariate and multivariate analyses.

Results: The cumulative incidence rates of HCC were 14%, 29% and 48% at 5, 10 and 15 years after liver biopsy, respectively. Multivariate analysis identified high viral load, together with age and severe fibrosis, as independent and significant risk factors ($P = 0.045$, 0.047 and 0.013, respectively) for HCC.

Conclusions: The present findings indicate that high viral load is a risk factor for HCC in patients with chronic HBV infection. Patients with a high HBV viral load should be carefully monitored for HCC.

Key words: hepatitis B virus, hepatitis B virus DNA, hepatocellular carcinoma, risk factor.

INTRODUCTION

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV). Complications of chronic hepatitis B, such as cirrhosis, hepatocellular carcinoma (HCC) and end-stage liver disease, account for approximately 1 million deaths each year.[1] Analysis of the risk factors for the development of HCC in patients with chronic HBV has been performed in several studies, and factors such as aging,[2–4] sex,[4] total alcohol intake,[2,4] nucleolar hypertrophy,[5] cirrhosis[6] and the presence of hepatitis B early antigen (HBeAg)[7] have been associated with HCC. A recent study has indicated that interferon (IFN) treatment can reduce the incidence of HCC.[6,8] The identification of additional variables associated with changes in the risk of developing HCC is of particular importance in the optimization of preventive medicine programs.

According to previous studies, seroconversion of HBeAg to antibody to HBeAg (anti-HBe) is believed to result in a decrease in viral load and to indicate a favorable outcome in these patients.[9–13] Furthermore, it has been reported that an increased risk of HCC is associated with HBeAg-positive patients.[7] Recent studies have indicated that the serum level of HBV-DNA is correlated with the progression of the disease.[14] However, it remains unclear if the serum level of HBV-DNA is a risk factor for hepatocarcinogenesis. In the current study, univariate and multivariate analyses of the risk factors, including the serum level of HBV-DNA, were carried out in 73 patients with chronic HBV infection.

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METHODS

Patients

Liver biopsies were obtained from 607 patients at the Nagasaki University Hospital (Nagasaki, Japan) between January 1980 and December 1999. Of these 607 patients, 47 were diagnosed with HCC based on histopathological examination of their tumor tissue, and/or by ultrasonography or computed tomography (CT). Of the 560 patients who had no detectable HCC, 181 were diagnosed with chronic hepatitis or cirrhosis and were confirmed to be negative for the antibody to HCV (anti-HCV), but positive for hepatitis B surface antigen (HBsAg). Of these 181 patients, 73 were followed at the Nagasaki University Hospital for more than 6 months and were enrolled in this study. Patients who had other causes of liver disease, such as primary biliary cirrhosis or autoimmune hepatitis, were excluded from the study. The 73 patients who were enrolled in the study fulfilled the following inclusion criteria: (i) absence of HCC at the time of liver biopsy; (ii) positive serological test for HBsAg; (iii) negative for anti-HCV; (iv) negative for autoantibodies, such as antinuclear antibody and antimitochondrial antibody; and (v) follow up for more than 6 months after the liver biopsy. At the time of liver biopsy, information on alcohol drinking habits was obtained through an interview conducted by the physicians. Habitual drinking was defined as an average daily consumption of an amount equivalent to 80 g/day of pure ethanol over a period of more than 5 years. Informed consent was obtained from each patient at the time of liver biopsy.

Serological tests

After informed consent was obtained, a serum sample was taken from each patient at the time of liver biopsy and was stored at −40°C for subsequent analysis of viral markers. The HBsAg, HBeAg and anti-HBe were assayed using commercially available radioimmunoassay kits (Dainabot, Tokyo, Japan). Anti-HCV was determined using a second- or third-generation ELISA (Ortho Diagnostics Systems, Tokyo, Japan). Serum HBV-DNA was detected by the transcription-mediated amplification (TMA) method, as described previously[15] and was expressed as the logarithm of the genome equivalent per milliliter (LGE/mL). The detection limit of this method is 3.7 LGE/mL. A value of 0.7 milliequivalents per milliliter (mEq/mL), the detection limit of HBV-DNA using a branched DNA assay, corresponds to that of 5.8 LGE/mL using the TMA method. Analysis of all viral markers, including serum HBV-DNA, was carried out on the serum sample taken from each patient at the time of liver biopsy.

Follow up of patients

Clinical evaluation and biochemical tests were carried out every 1–3 months. Ultrasonography or CT of the liver was carried out at least every 3–6 months. Diagnosis of HCC was based on the histopathological observation of tumor tissue or on characteristic signs in the ultrasonography, CT and hepatic arteriography. The endpoint used in the current study was the appearance of HCC and the reference date used was 31 December 2000. The number of patients who did not develop HCC for 5, 10 and 15 years after liver biopsy were 44, 24 and 10, respectively. A total of 23 patients were followed until the endpoint of the study and the average observation period was 89.6 months (7.4 years).

Histopathological examination of the liver

Liver biopsy specimens were fixed in 10% formalin, embedded in paraffin, cut to a thickness of 4 μm and stained with HE and Azan. All liver tissue specimens were evaluated by one pathologist (KT) who was unaware of the patient's clinical condition. Liver histology was evaluated according to the degree of fibrosis and necroinflammatory activity. The extent of fibrosis (staging) and the degree of necroinflammatory activity (grading) were classified according to Desmet *et al.* as follows: F1 (periportal expansion) and F2 (portoportal septa) were categorized as mild fibrosis, whereas F3 (portocentral linkage or bridging fibrosis) and F4 (cirrhosis) were categorized as severe fibrosis.[16] In terms of necroinflammatory activity, A1 represented mild activity and A2 or A3 represented severe activity.

Statistical analysis

Data were expressed as the mean ± SD for continuous variables and as counts for categorical variables. Continuous and categorical variables were compared using the Student's *t*-test and the χ^2 test, respectively. Cumulative incidence curves were determined using Kaplan–Meier analysis and the differences between groups were assessed using the log-rank test. Univariate and multivariate analyses of the risk ratios for the occurrence of HCC were studied using a Cox proportional-hazards regression analysis. Factors examined included age, sex, habitual drinking, serum ALT level, serum HBV-DNA level, IFN treatment during the follow-up period, histopathological staging (mild fibrosis or severe fibrosis), and histopathological grading (mild activity or severe activity). All *P*-values were two-tailed, and $P < 0.05$ was considered to be significant. The statistical analysis was performed using StatView version 5.0 (SAS Institute, Cary, NC, USA).

RESULTS

Patient characteristics

Table 1 shows the clinicopathological features of patients upon entry into the study. The study included 58 men and 15 women with a mean age of 39 years (range 16–68 years), and also included five patients (7%) who were habitual drinkers. Measurement of

Table 1 Clinical, laboratory and histological characteristics of the 73 patients

Male/female (%)	58/15 (79/21)
Mean age (years ± SD)	39.4 ± 11.7
Habitual heavy drinking (%)†	5 (7)
Diabetes (%)	7 (10)
Alanine aminotransferase (IU/L; mean ± SD)	170.7 ± 302.3
γ-Glutamyltransferase (IU/L; mean ± SD)	66.3 ± 66.6
Hepatitis B virus DNA (LGE/mL)	6.08 ± 1.68
Hepatitis B early antigen (positive/negative, %)	47/26 (64/36)
Interferon (yes/no)	7/66
Stage of fibrosis (%)	
1	17 (23)
2	13 (18)
3	14 (19)
4	29 (40)
Grade of inflammation (%)	
1	30 (42)
2	39 (53)
3	4 (5)

†Habitual drinking was defined as an average daily consumption of an amount equivalent to 80 g/day of pure ethanol over a period of more than 5 years. LGE, logarithm of the genome equivalent.

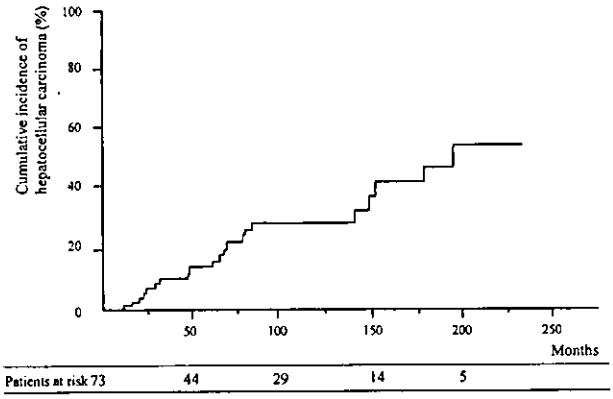

Figure 1 Cumulative incidence of hepatocellular carcinoma (HCC) in 73 patients with chronic hepatitis B.

HBV-DNA was achieved for 63 patients and the mean level of serum HBV-DNA was 6.08 LGE/mL (range <3.7–8.6 LGE/mL). Forty-seven patients (64%) were HBeAg-positive and 26 patients were HBeAg-negative. During the follow-up period, six of the 73 patients received IFN treatment. The numbers of patients with histopathological staging of F1, F2, F3 and F4 were 17 (23%), 13 (18%), 14 (19%) and 29 (40%), respectively. The numbers of patients with histopathological grading of A1, A2 and A3 were 30 (42%), 39 (53%) and four (5%), respectively.

Cumulative incidence of hepatocellular carcinoma

During the observation periods, 21 of the 73 patients (29%) developed HCC. Diagnosis of HCC was based on tumor tissue histopathological findings for eight patients, and on the characteristic appearance of images from ultrasonography, CT and/or hepatic arteriography for 13 patients. As shown in Figure 1, the cumulative incidence rates of HCC were 14%, 29% and 48% at 5, 10 and 15 years after liver biopsy, respectively.

Univariate analysis of clinicopathological factors influencing the development of hepatocellular carcinoma

To determine the factors that could influence the development of HCC, a Cox proportional-hazards regression analysis was carried out. Based on a univariate analysis of eight variables, the following three factors significantly influenced the incidence of HCC: age at the time of liver biopsy (relative risk 12.82; 95% confidence interval [CI], 1.72–100.00; $P = 0.013$); histopathological staging (severe fibrosis: relative risk 6.80; 95% CI, 1.59–3.53; $P = 0.010$) and; HBV-DNA level (high serum HBV-DNA level: relative risk 3.44; 95% CI, 1.07–7.46; $P = 0.035$) (Table 2).

Multivariate analysis of clinicopathological factors influencing the development of hepatocellular carcinoma

A multivariate analysis of the determinants of HCC was also applied using a Cox regression model. Age at the time of liver biopsy, histopathological staging and HBV-DNA level were identified as statistically independent risk factors (relative risk 8.20; 95% CI, 1.03–66.67; $P = 0.047$; relative risk 7.87; 95% CI, 1.54–40.00; $P = 0.013$; relative risk 3.08; 95% CI, 1.03– 9.17; $P = 0.045$, respectively) (Table 2). Figure 2 shows the cumulative incidence of HCC based on the HBV-DNA level. The cumulative incidence rate of HCC in 28 patients with a high HBV-DNA level (≥6.0 LGE/mL) was significantly higher than that in 35 patients with a low HBV-DNA level (<6.0 LGE/mL; $P = 0.0285$).

Clinicopathological findings in patients according to viral load

Table 3 shows the clinicopathological findings in patients according to viral load. Viral load is closely related to serum ALT level and the degree of necroinflammatory activity. Serum levels of HBV-DNA in patients with HCC who were HBeAg-positive were much higher than those in patients who were HBeAg-negative (median 6.4 LGE/mL *vs* 5.5 LGE/mL; $P = 0.032$; Fig. 3).

Table 2 Univariate and multivariate analysis of the risk ratios (RR) for hepatocellular carcinoma

	Univariate		Multivariate	
	RR (95% CI)	*P*-value	RR (95% CI)	*P*-value
Age (compared with <35 years)	12.82 (1.72–100.00)	0.013*	8.20 (1.03–66.67)	0.047*
Sex (compared with female)	2.10 (0.80–5.53)	0.134	2.28 (0.53–9.84)	0.269
Habitual heavy drinking† (compared with no drinking)	1.32 (0.17–10.10)	0.787	1.96 (0.22–17.24)	0.544
Alanine aminotransferase (compared with <80 IU/L)	2.18 (0.85–5.64)	0.107	2.72 (0.73–10.15)	0.136
Interferon (compared with no therapy)	2.20 (0.29–16.56)	0.444	1.78 (0.21–15.06)	1.776
Hepatitis B virus DNA (compared with <6.0 LGE/mL)	3.44 (1.07–7.46)	0.035*	3.08 (1.03–9.17)	0.045*
Staging (compared with mild)	6.80 (1.59–29.41)	0.010*	7.87 (1.54–40.00)	0.013*
Grading (compared with mild)	1.44 (0.59–3.53)	0.423	1.54 (0.45–5.21)	0.491

*Statistically significant. †Habitual drinking was defined as an average daily consumption of an amount equivalent to 80 g/day of pure ethanol over a period of more than 5 years. CI, confidence interval; LGE, logarithm of the genome equivalent.

Table 3 Clinicopathological findings in patients according to viral load

	Viral load		
Characteristic	High (≥6.0 LGE/mL)	Low (<6.0 LGE/mL)	*P*-value
Age (years; mean ± SD)	38 ± 10	40 ± 12	0.542
Sex (male/female)	8/27	5/23	0.625
Habitual heavy drinking (yes/no)†	3/32	2/26	0.835
Alanine aminotransferase (≥80/<80 IU/L)	22/13	9/19	0.015*
Staging (mild/severe)	12/23	15/13	0.124
Grading (mild/severe)	11/24	16/12	0.040*
Interferon (yes/no)	5/30	1/27	0.130

*Statistically significant. †Habitual drinking was defined as an average daily consumption of an amount equivalent to 80 g/day of pure ethanol over a period of more than 5 years.

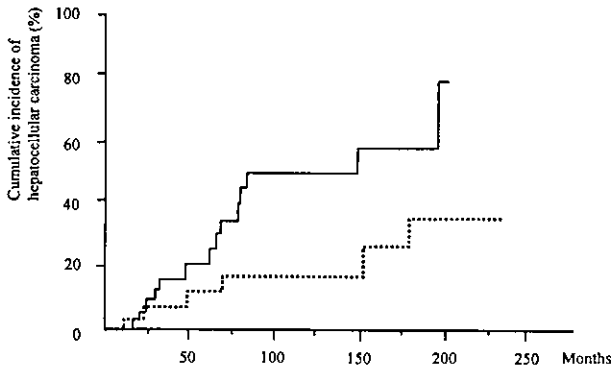

Figure 2 Cumulative incidence of hepatocellular carcinoma (HCC) based on the hepatitis B virus (HBV)-DNA levels in patients with chronic hepatitis B. The cumulative incidence of HCC in (—) 28 patients with high viral load (≥6.0 LGE/mL) was significantly higher than in (---) 35 patients with low viral load (<6.0 LGE/mL; $P = 0.0285$).

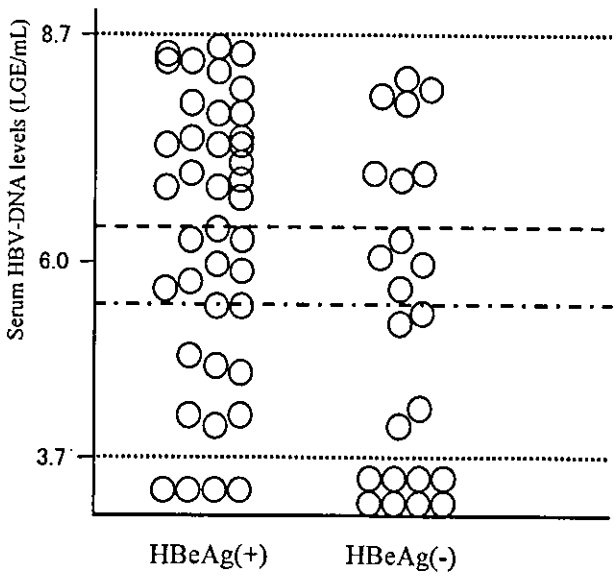

Figure 3 Relationship between the presence or absence of hepatitis B early antigen (HBeAg) and hepatitis B virus (HBV)-DNA levels. Mean serum level of HBV-DNA in patients who were HBeAg-positive was much higher than the value in those patients who were HBeAg-negative (6.4 ± 1.6 LGE/mL *vs* 5.5 ± 1.8 LGE/mL; $P = 0.032$).

DISCUSSION

To identify the relationship of HCC with HBV-related chronic liver disease, including cirrhosis, we investigated the development of HCC in patients infected with

HBV and assessed the associated risk factors for HCC. The results may prove useful in the development of strategies in preventive medicine programs for HCC, and also for identifying screening approaches for the early detection of HCC.

In the current study, the cumulative incidence of HCC in 73 patients with HBV-associated liver disease was almost the same as that found in a previous study.[2] Among the risk factors for HCC that were analyzed, a high viral load of HBV-DNA, together with age and histological fibrosis, were found to be linked to the occurrence of HCC, based on both univariate and multivariate analyses.

It has been reported that the viral load of HBV-DNA is often associated with the presence or absence of HBeAg.[9,11,17] We also found that the viral load of the HBeAg-positive group was higher than that of the HBeAg-negative group (Fig. 3) and that viral load is closely related to the serum ALT level and the grade of histological inflammation (Table 3). These results are in accordance with other published studies.[9]

During the course of chronic HBV infection, the presence of HBeAg is often associated with active and continuing liver disease, whereas HBeAg seroconversion to anti-HBe often coincides with loss of serum HBV-DNA, normalization of liver biochemistry, clinical remission and subsidence of hepatic inflammatory activity.[12,13,18–21] Another study suggests that spontaneous HBeAg seroconversion confers a favorable long-term outcome in the majority of patients. However, HBeAg reversion or HBeAg-negative hepatitis may develop in some patients who subsequently show an increased risk of cirrhosis or HCC, as compared with patients with sustained remission.[22]

Most patients with HBeAg-negative, anti-HBe-positive and HBV-DNA positive chronic hepatitis B have HBV variants with mutations in the precore or core promoter region. In patients with HBV variants, progressive liver damage occurs in parallel with relatively high levels of viremia.[14,22] Ohkubo *et al.* reported that the baseline level of serum HBV-DNA did influence the survival time of patients with HBV-associated HCC, even for patients with HBeAg-negative disease.[23] However, the current study did not show a statistically significant difference in the occurrence of HCC between the high and low viral load groups, perhaps because of the small number of HBeAg-negative patients ($P = 0.13$, data not shown). In HBV-related cirrhotic patients, HBV-DNA was the strongest predictive factor for the development of HCC[24] and a high viral load was found to be associated with a high recurrence rate of HCC after surgical resection in patients with HBV.[9] Thus, it is conceivable that patients with a high viral load may have a high potential for hepatocarcinogenesis.

Chronic infection with HBV is involved in hepatocarcinogenesis by induction of a long-term process of liver cell necrosis and inflammation,[25,26] as well as by *cis*- and *trans*-activation effects on cellular genes.[27] However, there are many theories to explain hepatocarcinogenesis in HBV infection, and the detailed mechanism of hepatocarcinogenesis remains unclear. Even though HCC develops in some HBV-infected patients in the absence of cirrhosis, a direct carcinogenic role of HBV has been suggested. Integration of the HBV genome into cellular DNA has been found in the majority of HBV-induced HCC patients.[28] In addition, it has been recently reported that, compared with hepatitis C patients, cirrhotic patients infected with HBV are at a higher risk of developing an infiltrative and more aggressive type of HCC that is independent of duration or stage of cirrhosis and may reflect more direct viral carcinogenesis.[29]

It is generally agreed that IFN therapy is effective in patients with chronic HBV infection in terms of virological and histological remission.[6,30,31] Antiviral treatment with nucleoside analogs, such as lamivudine or adefovir, has recently been implemented in patients with HBV. In clinical trials, treatment with lamivudine or adefovir improved histological liver abnormalities, reduced serum HBV-DNA levels and normalized ALT levels.[32–36] In the current study, it was found that high viral load is a risk factor for hepatocarcinogenesis in patients with chronic HBV. However, it remains unclear whether antiviral therapeutic agents, such as IFN or nucleoside analogs, prevent HCC with chronic HBV because of the limited number of clinical studies. It has been suggested that the integration of viral DNA into the host genome is essential for carcinogenesis through the activation or suppression of several genes,[37] and that treatment with IFN or nucleoside analogs cannot affect viral DNA that has already been integrated. To clarify the effectiveness of antiviral therapy in carcinogenesis further studies and follow up are required.

In the current study, viral load was found to be an independent risk factor for HCC in patients with chronic HBV. Although the independent role of the level of viremia observed in the current study must be validated by further investigations, more intensive monitoring of HCC should be considered in chronic HBV patients with a high viral load.

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LIVER

Bone marrow engraftment in a rodent model of chemical carcinogenesis but no role in the histogenesis of hepatocellular carcinoma

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Background and aim: Recent studies indicated that hepatic stem cells in the bone marrow could differentiate into mature hepatocytes, suggesting that bone marrow cells could be used for replacement of damaged hepatocytes in a variety of liver diseases. Hepatocellular carcinoma (HCC) is thought to arise from hepatic stem cells. In this study, we investigated the malignant potential of hepatic stem cells derived from the bone marrow in a mouse model of chemical hepatocarcinogenesis.

Methods: Bone marrow cells were obtained from the male β-galactosidase (β-gal) transgenic mouse and transplanted into female recipient mice. Hepatocarcinogenesis was induced by a year of treatment with diethylnitrosamine and phenobarbital (NDEA/PB). One year later, the liver was removed from each treated mouse and evaluated by x-gal staining, immunohistochemistry, and fluorescence in situ hybridisation (FISH).

Results: Forty per cent of recipient mice survived and developed multiple HCC. Clusters of β-gal positive mature hepatocytes were detected sporadically in the entire liver of NDEA/PB treated mice who underwent bone marrow transplantation (BMT) with while no such hepatocytes were identified in the liver of BMT mice that were not treated with NDEA/PB. The Y chromosome was detected with the same frequency as the donor male liver in clusters of β-gal positive mature hepatocytes by FISH. However, no HCC was positive for β-gal or the Y chromosome. Immunohistochemically, β-gal positive mature hepatocytes did not express CD34 or α-fetoprotein.

Conclusions: Our results suggest that hepatic stem cells derived from the bone marrow have low malignant potential, at least in our model.

Although patients at high risk of hepatocellular carcinoma (HCC) are well defined in the clinical setting, the process of hepatocarcinogenesis is not well understood.[1 2] Hepatitis B or C virus can induce chronic hepatitis and potentially results in liver cirrhosis and HCC, and patients with these viral infections are the most frequent among HCC patients.[3] However, the consequences of viral infection in the process of hepatocarcinogenesis are not clearly understood. In this regard, the original cell that develops into a cancerous cell in HCC is debated.[4] Two cell lineages have been considered as candidates: the first is the hepatic stem cell and the second is the mature hepatocyte. However, there is no clear evidence on which cell is directly involved in the development of HCC.[5 6]

Extensive studies have been conducted on the hepatic stem cell but the results are controversial. Several candidate hepatic stem cells have been described in a rodent experimental model (including oval cells, liver epithelial cells, and small hepatocytes).[7–9] Previous studies indicated that bone marrow cells can differentiate into oval cells in rodents and that a similar process could possibly take place in humans.[10–12] If this scenario is correct, isolation of hepatic stem cells from bone marrow cells could be a valuable strategy for future replacement therapy of damaged or malfunctioned hapatocytes.[10] However, the safety and efficacy of hepatic stem cells derived from bone marrow cells should be adequately confirmed before any such therapies are tested in humans.

In an attempt to assess hepatic stem cells, in the present study we investigated the malignant potential of hepatic stem cells derived from bone marrow in vivo. To identify hepatic stem cells, bone marrow cells of β-galactosidase (β-gal) transgenic male mice were transplanted into recipient female mice. After bone marrow transplantation (BMT), HCC was induced in the recipients by chemical hepatocarcinogenic compounds and the presence of β-gal and the Y chromosome were evaluated in HCC.

MATERIALS AND METHODS

Bone marrow transplantation

Six week old male B6-129S-Gtrosa and female B6-129-F2 mice were purchased from Jackson Laboratory (Bar Habor, Maine, USA). B6-129S-Gtrosa mice express transgenic β-gal in the whole body and B6-129-F2 mice are the parental strain of B6-129S-Gtrosa.[13] Animal experiments were performed in accordance with institutional guidelines, and the study was approved by the ethics committee of Nagasaki University. BMT was performed as reported previously.[14] Briefly, the thigh was removed from a dead male B6-129S-Gtrosa, and bone marrow cells were harvested and suspended in phosphate buffered saline at a density of 1×10^7 cells/ml. Female B6-129-F2 mice were irradiated sublethally and 5×10^6 of the above bone marrow cells were injected via the tail vein. To confirm successful transplantation of donor bone marrow cells, a sample of recipient bone marrow cells was obtained from B6-129-F2 mice after sacrifice and examined for x-gal staining.

Abbreviations: AFP, α-fetoprotein; β-gal, β-galactosidase; BMT, bone marrow transplantation; FISH, fluorescence in situ hybridisation; HCC, hepatocellular carcinoma; NDEA/PB, diethylnitrosamine/phenobarbital; H&E, haematoxylin-eosin

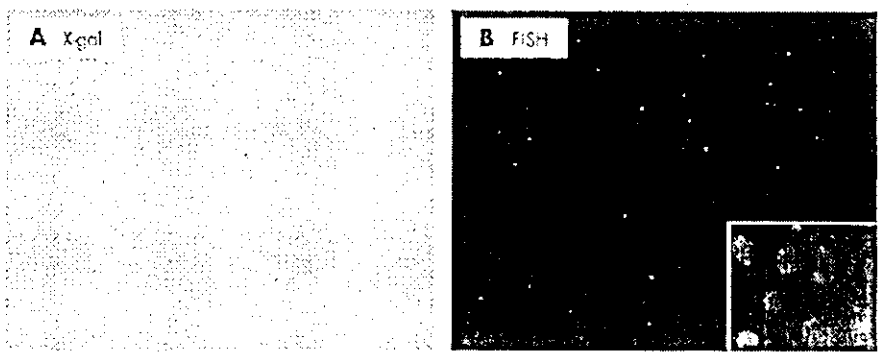

Figure 1 X-gal staining and fluorescence in situ hybridisation (FISH) for the Y chromosome in the liver of the donor mouse. To confirm expression of β-galactosidase and status of the Y chromosome in donor mouse, x-gal staining and FISH were performed. (A) X-gal staining (magnification ×100). (B) FISH for the Y chromosome (magnification ×100). Inset; high magnification of FISH with counterstaining by DAPI (magnification ×400).

Chemically induced hepatocarcinogenesis

To induce HCC, two weeks after BMT, 80 mg/l diethylnitrosamine (NDEA; Sigma, St Louis, Missouri, USA) was added to the drinking water of B6-129-F2 recipient mice for six weeks to initiate the process of hepatocarcinogenesis. This was followed by administration of 700 mg/l phenobarbital (PB; Wako, Tokyo, Japan) as a tumour promoting agent, until sacrifice. Ten bone marrow transplanted and 10 non-transplanted mice were each treated with NDEA/PB, and four mice who underwent BMT but not NDEA/PB treatment and four mice who received no treatment were maintained under the same conditions as controls.

X-gal staining

One year after the start of NDEA, mice who survived were sacrificed and the liver and bone marrow harvested. The liver was fixed in 10% formalin for 24 hours and paraffin embedded. Simultaneously, fresh frozen sections were also prepared. Routine histology was performed with haematoxylin-eosin (H&E) staining of both formalin fixed/paraffin embedded and fresh frozen sections. To identify bone marrow derived cells in the liver specimens, every fifth slide of 5 μm thick serial sections (total 125 slides) was stained for x-gal. X-gal staining kit (Gene Therapy Systems, San Diego, California, USA) was used with fresh frozen sections of the liver removed from each group according to the instructions provided by the manufacturer.

Immunohistochemistry

Immunohistochemistry was performed using antimouse CD34 or antimouse α-fetoprotein (AFP) antibody as the primary antibody. Tissue samples of the fresh frozen liver sections were cut into 5 μm thick sections and mounted on aminopropyltriethoxysilane coated glass slides. The streptavidin-biotin method (Histofine Staining Kit; Nichirei Company, Tokyo, Japan) was used for immunohistochemical detection, as described previously.[15] X-gal and immunohistochemically treated slides were counterstained with haematoxylin. Numbers of clusters or cells positively stained in each section were counted and mean (SD) values were calculated for each sample.

Table 1 Survival rate and success of BMT

Treatment	Survival (n)	β-gal positive/negative bone marrow cells
(1) BMT(−), NDEA/PB(−)	100% (4/4)	0%
(2) BMT(+), NDEA/PB(−)	100% (4/4)	95%
(3) BMT(−), NDEA/PB(+)	50% (5/10)	0%
(4) BMT(+), NDEA/PB(+)	40% (4/10)	91%

BMT, bone marrow transplantation; NDEA, diethylnitrosamine; PB, phenobarbital; β-gal, β-galactosidase.

Fluorescence in situ hybridisation

Because BMT was performed from male donor mice to female recipient mice, the transplanted bone marrow derived cells could be recognised in the recipient by the presence of the Y chromosome in the nucleus. Therefore, fluorescence in situ hybridisation (FISH) for the mouse Y chromosome was conducted to detect the transplanted bone marrow derived cells. Liver specimens of fresh frozen sections were pretreated using the Pretreatment Reagent Kit (Vysis, Downers Grove, Illinois, USA) according to the instructions supplied by the manufacturer, and hybridised with a mouse Y chromosome probe (Cambio, Cambridge, UK). Treated slides were observed under fluorescent microscopy.

RESULTS

X-gal staining and FISH of donor mice

In this study, we used the male B6-129S-Gtrosa mouse as a donor. It has been reported previously that this mouse expresses β-gal throughout the whole body.[15] To confirm this, we performed x-gal staining and FISH for the Y chromosome in the liver. All hepatocytes were positive for x-gal staining (fig 1A) and 98% of bone marrow cells were x-gal stained (data not shown). FISH revealed that 28% of hepatocyte nuclei were positive for the Y chromosome (fig 1B). A positive FISH signal was detected in the nucleus which was confirmed by counterstaining with DAPI (fig 1B, inset). In addition, no nucleus showed two or more signals.

Survival rate

We first evaluated the survival rate of mice that underwent BMT and/or NDEA/PB treatment (table 1). All (100%, 4/4 each) control mice and mice that underwent BMT only were still alive at the end of the study. However, only five of 10 (50%) mice in the NDEA/PB treated group that did not undergo BMT were still alive at the end of the one year study period. The survival rate of mice subjected to BMT and NDEA/PB treatment was the lowest (4/10 mice, 40%).

Bone marrow transplantation

To confirm bone marrow replacement with β-gal gene transduced cells, bone marrow cells derived from each group of recipient mice were subjected to x-gal staining (fig 2A, 2B). Red blood cells were excluded by careful microscopic observation and only nuclear cells were examined. No x-gal stained bone marrow nuclear cells were found in mice that did not receive BMT. In contrast, 91–95% of bone marrow

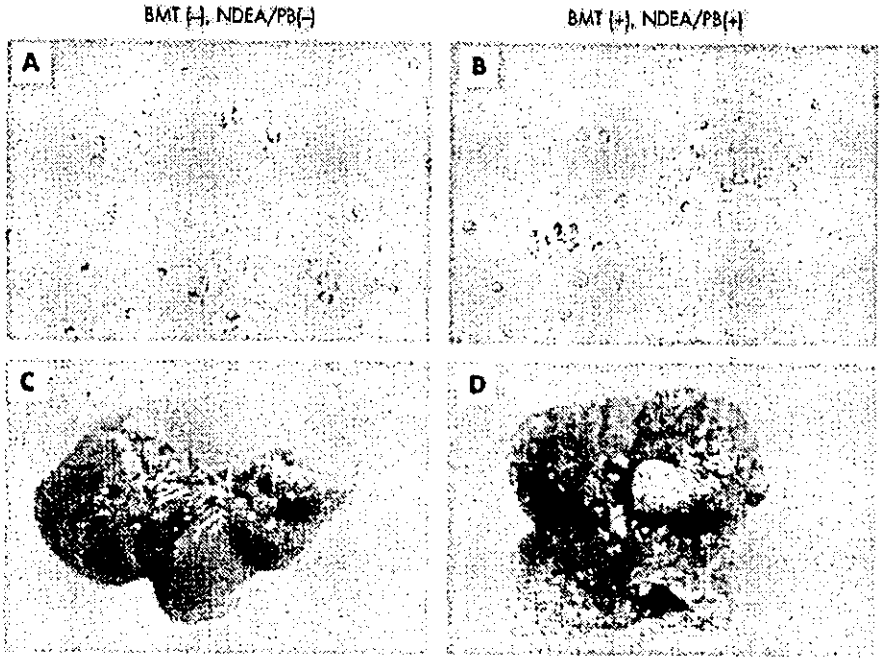

Figure 2 X-gal staining of bone marrow cells and hepatocellular carcinoma development in mice that underwent bone marrow transplantation (BMT) and diethylnitrosamine/phenobarbital (NDEA/PB) treatment. One year after the start of treatment, bone marrow cells were removed from each mouse and stained with x-gal. β-Gal positive cells appear as blue stained cells. Representative photographs are shown. (A) Bone marrow cells derived from a non-treated control mouse. (B) BMT and NDEA/PB treated mouse. (C) The liver removed from a non-treated control mouse. (D) BMT and NDEA/PB treated mice (magnification A and B, ×200).

cells were x-gal stained in mice that received BMT with or without NDEA/PB treatment (table 1). These results indicated successful BMT in this study.

Histopathological analysis of the liver of recipient mice

NDEA/PB treatment resulted in the appearance of multiple macroscopic tumours in the liver after one year of treatment in both BMT and non-BMT mice (fig 2C, 2D). H&E stained sections of these tumours confirmed they were HCC (see fig 6A), based on the criteria defined previously.[16] No other types of liver tumours, such as hepatoblastoma or cholangiocellular carcinoma, were noted in our experiment. Apart from HCC, abundant denatured hepatocytes, oval cells, cysts, and bile duct proliferation were observed in NDEA/PB treated mice compared with control livers.

Phenotypic analysis by x-gal staining, immunohistochemistry, and FISH

In a preliminary study, we sacrificed two BMT mice and liver samples were removed before NDEA/PB treatment. In these samples, β-gal positive hepatocytes were not identified (data not shown). Furthermore, no β-gal positive mature hepatocytes were detected in the livers of BMT mice that were not treated with NDEA/PB one year after BMT except for hematic cells in blood vessels (fig 3). However, x-gal staining of fresh frozen liver samples of four BMT mice treated with NDEA/PB showed sporadic clusters of β-gal positive mature hepatocytes (fig 4A, 4B). The number of these clusters in four mice was mean 0.18 (SD 0.10) in 53 (15) mm^2 sections (0.34 (0.10) cluster/cm^2, n = 125 slides). Furthermore, 17 (4.2) hepatocytes were present within the diameter of a cluster. Immunohistochemical analysis showed that β-gal positive

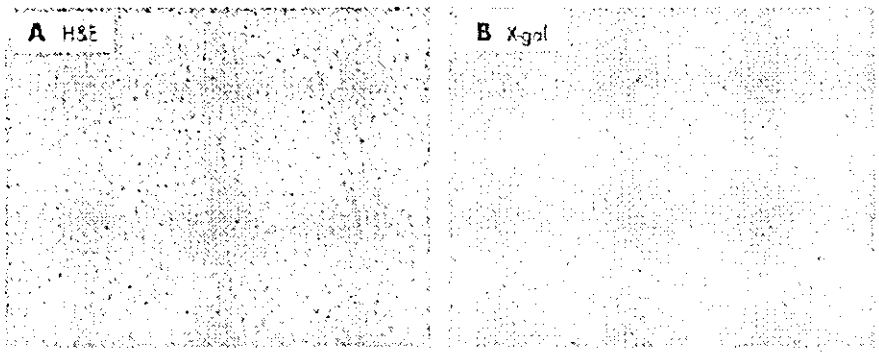

Figure 3 Haematoxylin-eosin (H&E) and x-gal stained liver section obtained from a mouse who underwent bone marrow transplantation (BMT) but not diethylnitrosamine/phenobarbital treatment. One year after BMT, the mouse was sacrificed and the liver removed and stained, as described in materials and methods. (A) H&E staining. (B) X-gal staining (magnification ×100).

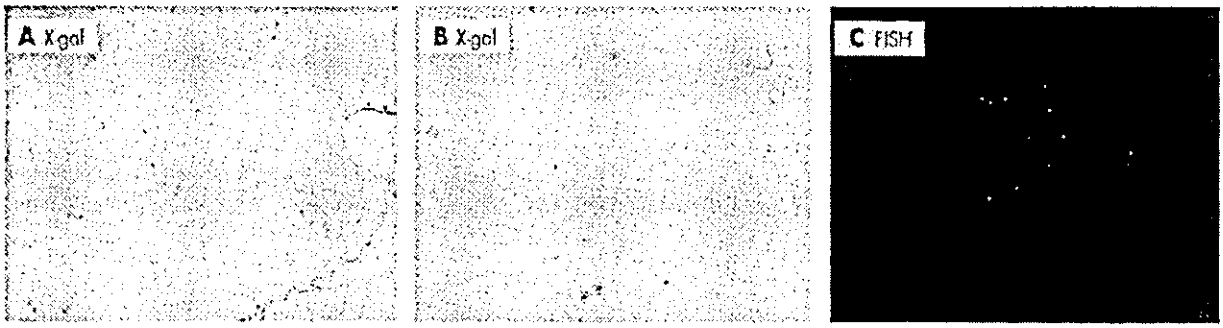

Figure 4 Appearance of β-galactosidase (β-gal) positive cell clusters and confirmation of the Y chromosome. (A) Numbers of β-gal positive cell clusters were counted in each x-gal stained slide examined under low magnification (×40). (B) Representative photograph of β-gal positive cell cluster under high magnification (×100). (C) Fluorescence in situ hybridisation (FISH) was performed as described in materials and methods to confirm the cell origin (magnification ×100).

mature hepatocytes were negatively stained for CD34 and AFP (fig 5). In addition, FISH showed that β-gal positive mature hepatocytes were positive for the Y chromosome with the same frequency as the liver of donor male mice (26%, fig 4C). However, no HCC was positive for x-gal staining or FISH for the Y chromosome (fig 6, n = 10 tumours).

DISCUSSION

The liver is classified as a conditionally renewing tissue and under normal circumstances nearly all hepatocytes proliferate quiescently, with only 0.3–0.5% of cell showing active cell division.[17] Under normal physiological conditions, there is no need for hepatic stem cells, and they only become evident on demand (for example, in circumstances associated with severe hepatic liver damage and coexistent impaired hepatocyte division). Previous studies demonstrated that oval cells, which are considered hepatic stem cells, appear after chemically induced liver injury.[18] Although the oval cell clearly appears in such conditions, its precise origin and existence under normal conditions remains unclear. Recent studies reported that bone marrow cells could differentiate into oval cells.[12 19] This finding indicates not only the origin of the oval cell but also the feasibility of in vivo labelling, at least in part, of oval cells.

In this study, we labelled bone marrow cells by β-gal staining. All hepatocytes were positive for β-gal in donor mice. Also, sensitivity of FISH for the Y chromosome was almost the same as that reported previously[11 12] while this sensitivity was inadequate for the detection of donor cells. Thus we believe that our methods for tracing bone marrow cells of donor mice were acceptable in the present study. After BMT, clusters of β-gal positive cells appeared only in BMT mice treated with NDEA/PB and not in BMT mice who did not receive NDEA/PB. These results suggest that in our

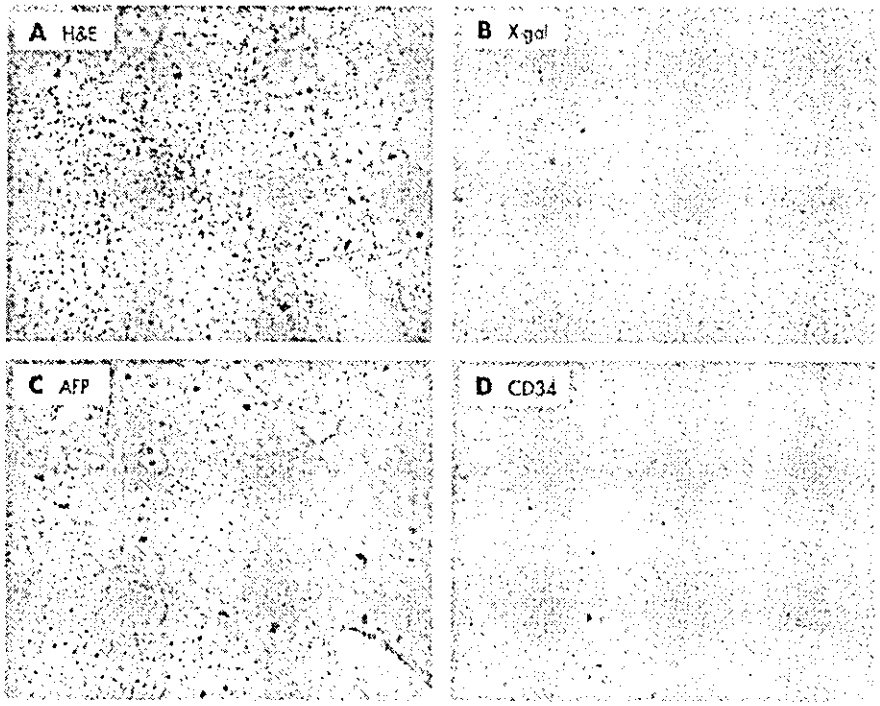

Figure 5 Immunohistochemical analysis of β-galactosidase (β-gal) positive cell clusters in bone marrow transplantation and diethylnitrosamine/phenobarbital treated mice. The phenotype of the β-gal positive cluster was determined by immunostaining. (A) Haematoxylin-eosin (H&E) staining. (B) X-gal staining. (D) Immunostaining with antimouse CD34 antibody. (C) Antimouse α-fetoprotein (AFP) (magnification ×100).

Figure 6 Histopathological analysis and x-gal staining of the tumour. Hepatocellular carcinoma (HCC) was induced by one year of treatment with diethylnitrosamine/phenobarbital (NDEA/PB), as described in materials and methods. Extracted livers were observed histopathologically under the microscope (×100). (A) Representative photograph of haematoxylin-eosin (H&E) stained liver section obtained from a bone marrow transplantation and NDEA/PB treated mouse. T, HCC tumour; N, adjacent normal liver tissue. (B) X-gal staining of the serial section without any counterstaining. β-Galactosidase positive cells appear as blue stained cells (×100). (C) Fluorescence in situ hybridisation (FISH) of the serial section (magnification ×100).

model, bone marrow cells can differentiate into mature hepatocytes under limited conditions. Bone marrow derived hepatic stem cells do not seem to be required for normal hepatocyte substitution. In addition, all hepatic stem cells derived from bone marrow cells may not have been labelled by this method and pre-existent hepatic stem cells, the origin of which is not clear, may be present in the liver. It has been reported that a stem cell of a particular tissue can differentiate into another tissue.[20 21] A stem cell network may exist in the whole body and involve each other in variant tissues. Recently, some investigators have reported that cell fusion between a hepatic lineage cell and a bone marrow cell was the source of bone marrow derived hepatocytes in the $FAH^{-/-}$ mouse model.[22 23] However, other studies have shown that such fusion does not always play a role in haematopoietic to epithelial cell engraftment.[24] Therefore, cell fusion may occur under specific conditions such as in the $FAH^{-/-}$ mouse model.[25] Indeed, in the present study, we found that in β-gal positive hepatocytes, no nucleus had two or more Y chromosomes by FISH. This finding indicates that transdifferentiation, rather than cell fusion, was the main process in our model.

In this study, our interest was focused on the original cell lineage of HCC. Two major hypotheses (the "stem cell theory" and the "hepatocytic theory") have been discussed for almost 20 years.[6 26 27] Debate has centred on whether hepatocytes in dysplastic nodules are responsible for HCCs through a process of dedifferentiation and proliferation, or whether oval cells are the prime targets for malignant changes after a differentiative "block", as proposed in some animal models.[7 28] Possible involvement of oval cells in hepatocarcinogenesis is based on the following: (1) massive existence of oval cells in an animal rodent hepatocarcinogenic model[29]; (2) development of HCC after transformation of oval cells[30 31]; and (3) occurrence of mixed hepatocellular and cholangiocarcinomatous tumours (oval cell exhibits bipotential developmental ability).[32 33] However, the relationship between oval cells and cancer is only circumstantial. In this study, no HCC was positive for β-gal, and all β-gal positive cell clusters were oval cells or mature hepatocytes at the point of sacrifice, even after long term carcinogenic induction. These results allow exclusion of the stem cell theory and acceptance of the hepatocytic theory in hepatocarcinogenesis. However, as all hepatic stem cells might not have been labelled by our method, as mentioned above, we cannot completely exclude the stem cell theory. Although our results may be limited to BMT mice treated with NDEA/PB, we can state that the malignant potential of the hepatic stem cell derived from bone marrow seems to be low. Patients at high risk of HCC are clearly defined in the clinical settings, and viral hepatitis and/or related cirrhosis are the most critical factors.[3 34] We selected the NDEA/PB model in the present study as it is a useful model of human hepatocarcinogenesis caused by viral hepatitis as nitrate and nitrosamine synthesis is increased in viral hepatitis.[35 36] Further studies are needed to clarify the precise interaction of bone marrow cells with hepatic regeneration and carcinogenesis using other animal models or human studies.

Liver transplantation has already been introduced for the treatment of patients with chronic or acute hepatic failure and congenital or malignant liver disease, and a large proportion of such patients have previously been reported to benefit from this procedure.[37] However, there are some problems with liver transplantation, including shortage of donors.[38] Cell based therapy is being developed for replacement of damaged or malfunctioned hepatocytes.[39] Bone marrow cells may potentially be used in cell based replacement therapy or gene delivery systems. Under these circumstances, our results indicate that stem cell therapy is safe.

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