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Applied nutritional investigation

Elevated serum tumor necrosis factor-α and soluble tumor necrosis factor receptors correlate with aberrant energy metabolism in liver cirrhosis

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Abstract

Objective: Protein–energy malnutrition is frequently observed in patients with liver cirrhosis and is associated with their poor prognosis. Tumor necrosis factor-α (TNF-α) is elevated in those patients and may contribute to the alterations of energy metabolism. Our aim was to characterize the aberrant energy metabolism in cirrhotic patients with regard to TNF-α.

Methods: Twenty-four patients (mean age 65 ± 6 y) with viral liver cirrhosis who did not have hepatocellular carcinoma or acute infections were studied. Twelve healthy volunteers were recruited after matching for age, gender, and body mass index with the patients and served as controls (59 ± 8 y). Serum levels of TNF-α, soluble 55-kDa TNF receptor (sTNF-R55), soluble 75-kDa TNF receptor (sTNF-R75), and leptin were determined by immunoassay. Substrate oxidation rates of carbohydrate and fat were estimated by indirect calorimetry after overnight bedrest and fasting.

Results: In cirrhotic patients, serum levels of TNF-α, sTNF-R55, and sTNF-R75 were significantly higher than those in the controls and correlated with the increasing grade of disease severity as defined by Child-Pugh classification. Serum leptin concentration was not different between cirrhotics and controls but correlated with their body mass index. The decrease in substrate oxidation rate of carbohydrate and the increase in substrate oxidation rate of fat significantly correlated with serum TNF-α, sTNF-R55, and sTNF-R75 concentrations.

Conclusion: Tumor necrosis factor-α might be associated with the aberrant energy metabolism in patients with liver cirrhosis.

Keywords: Liver cirrhosis; Tumor necrosis factor-α; Soluble 55-kDa tumor necrosis factor receptor; soluble 75-kDa tumor necrosis factor receptor; Leptin; Indirect calorimetry; Protein–energy malnutrition

Introduction

A significant proportion of patients with liver cirrhosis shows protein–energy malnutrition (PEM) [1,2], and PEM leads to poor prognosis in these cases [3–5]. Energy metabolism and nutritional status can be estimated, for example, by indirect calorimetry [5,6] and by anthropometry, such as triceps skinfold thickness (TSF) and arm muscle circumference (AMC) [7]. Impaired energy metabolism significantly correlates with a worse event-free survival of cirrhotics [5,7,8]. However, the mechanisms underlying energy malnutrition in cirrhosis have not been elucidated enough [2]. Candidate causes for this state include enhanced secretion of cytokines such as tumor necrosis factor-α (TNF-α) and adipokines represented by leptin in liver cirrhosis, because both induce anorexia and increase energy expenditure, leading to physical wasting [9–11].

Tumor necrosis factor-α, or cachectin, is a proinflammatory cytokine and is released mainly from monocytes and lymphocytes in response to inflammatory stimuli [12]. TNF-α is also postulated to play a role in the development of anorexia or physical wasting and to regulate energy

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Table 1
Baseline demographic characteristics, body composition, blood biochemistry, and calorimetric data in cirrhotic patients and control subjects*

	Controls ($n = 12$)	Cirrhosis ($n = 24$)	$P^{\dagger}$	Child A ($n = 9$)	Child B ($n = 9$)	Child C ($n = 6$)	$P^{\ddagger}$
Age (y)	58.5 (48–68)	64 (64–75)	0.31	63 (58–69)	67 (63–75)	61 (54–76)	0.10
Male/female	8/4	16/8	0.58	6/3	5/4	5/1	0.90
Height (cm)	168 (153–175)	159 (143–173)	<0.05	159 (145–170)	156 (143–166)	161 (144–173)	0.67
Weight (kg)	62 (54–66)	59 (47–87)	0.36	64 (48–74)	56 (39–67)	59 (47–87)	0.51
Body mass index (kg/m^2)	22.0 (20.8–24.3)	21.3 (17.3–26.9)	0.42	22.9 (19.0–26.9)	22.1 (17.0–29.0)	18.5 (17.7–22.5)	0.11
Arm muscle circumference (%)	101 (85–107)	96 (78–107)	0.59	104 (87–112)	91 (72–108)	95 (78–105)	<0.01
Triceps skinfold thickness (%)	139 (82–200)	92 (46–180)	<0.01	104 (84–181)	92 (46–138)	80 (55–168)	0.05
Total bilirubin (mg/dL)	0.7 (0.4–0.9)	1.4 (0.7–6.6)	<0.01	1.2 (0.7–1.9)	1.4 (0.4–2.5)	3.5 (1.4–6.5)	<0.01
Albumin (g/dL)	4.6 (4.2–4.8)	3.1 (2.4–4.4)	<0.01	3.3 (2.4–4.4)	3.0 (2.5–3.3)	2.8 (2.0–3.4)	0.07
Alanine aminotransferase (IU/L)	18 (8–25)	50 (22–140)	<0.01	71 (22–140)	39 (26–106)	46 (38–69)	0.10
Prothrombin time (%)	98 (94–102)	69 (30–150)	<0.01	83 (55–150)	62 (47–87)	50 (30–65)	<0.01
TNF-α (ng/L)	3.0 (2.0–3.5)	8.3 (5.0–19.0)	<0.01	6.6 (5.0–11.0)	9.4 (7.4–15.1)	12.3 (7.0–19.0)	<0.01
sTNF-R55 (ng/L)	1025 (773–1450)	2505 (1390–5000)	<0.01	1575 (1350–5000)	2680 (2000–5000)	3240 (2840–5000)	<0.01
sTNF-R75 (ng/L)	1805 (1460–2360)	4255 (1840–5000)	<0.01	3210 (1840–5000)	4900 (3920–5000)	4800 (4000–5000)	<0.01
Leptin (μg/L)	4.3 (3.2–5.4)	4.7 (1.5–17.5)	0.95	6.3 (2.9–17.5)	3.8 (1.5–13.9)	2.8 (1.7–8.3)	0.09
REE (kcal/d)	1330 (1140–1450)	1188 (892–1830)	0.41	1507 (981–1830)	1188 (1011–1826)	1655 (1011–1342)	0.36
BMR (kcal/d)	1355 (1163–1428)	1170 (1077–1760)	0.09	1250 (1110–1530)	1160 (850–1380)	1170 (1077–1760)	0.47
npRQ	0.91 (0.87–0.94)	0.84 (0.70–0.97)	<0.01	0.88 (0.84–0.97)	0.83 (0.77–0.86)	0.75 (0.70–0.80)	<0.01
CHO (%)	50.4 (42.0–56.3)	39.9 (18.4–61.6)	<0.01	42.3 (25.7–61.6)	39.7 (18.4–48.7)	25.4 (19.9–30.6)	<0.01
FAT (%)	29.1 (22.6–37.2)	37.5 (23.0–61.6)	<0.01	31.0 (25.9–53.9)	38.0 (23.0–55.5)	61.0 (45.9–61.6)	<0.01
PRO (%)	20.7 (11.6–26.4)	22.7 (5.2–32.3)	0.43	23.8 (11.8–33.0)	25.2 (5.2–32.3)	14.5 (12.9–23.5)	0.08

BMR, basal metabolic rate predicted by the Harris-Benedict formula; Child, Child-Pugh grade; CHO, substrate oxidation rate of carbohydrate; FAT, substrate oxidation rate of fat; PRO, substrate oxidation rate of protein; REE, resting energy expenditure; TNF-α, tumor necrosis factor-α; npRQ, non-protein respiratory quotient; sTNF-R55, soluble 55-kDa tumor necrosis factor receptor; sTNF-R75, soluble 75-kDa tumor necrosis factor receptor.

* Values are expressed as median (range).

† Between controls and cirrhosis by Fisher's exact test or Mann-Whitney U test.

‡ For distribution among Child-Pugh grades by chi-square test or one-way analysis of variance.

metabolism [9,10]. The actions of TNF-α are mediated by two distinct cell-surface receptors, soluble 55-kDa tumor necrosis factor receptor (sTNF-R55) and soluble 55-kDa tumor necrosis factor receptor (sTNF-R75) [12–14]. These two receptors also exist in soluble form after their extracellular domains have been cleaved proteolytically from the cell surface. These soluble receptors can block TNF-α activity by competing with cell-surface TNFR or prolong the biological effect of TNF-α as a buffer system. Because circulating TNFR levels remain elevated for a longer period than TNF-α, it is proposed that sTNFR levels may serve as a more sensitive means of monitoring the activity of the TNF-α system. In previous reports, serum TNF-α, sTNF-R55, and sTNF-R75 concentrations were elevated in cirrhotic patients [15,16], correlating with severity of liver damage [16–18]. However, little has been studied regarding the relation between TNF-α and alternations of energy metabolism in cirrhosis [19].

Leptin, a 16-kDa protein product by adipocytes, is postulated to regulate energy balance by suppressing appetite and increasing energy expenditure [11]. In several studies, serum leptin concentrations were elevated in cirrhotic patients, which suggested that leptin might play a role in the development of anorexia or physical wasting associated with cirrhosis [20–22].

Thus, in this study we aimed to test whether elevated serum concentrations of TNF-α, sTNF-R55, sTNF-R75, and leptin are associated with the aberrant energy metabolism in patients with liver cirrhosis.

Materials and methods

Patients

Twenty-four patients with liver cirrhosis (16 men and 8 women, mean age 65 ± 6 y) participated in this study. Liver cirrhosis was diagnosed by clinical and laboratory profiles and by histologic examination of liver biopsy specimens. Child-Pugh grade of disease severity [23] was A in nine cases, B in nine cases, and C in six cases. Hepatitis C virus was the cause of cirrhosis in all cases. Patients with physically detectable ascites or peripheral edema of moderate to severe grade [8] were excluded. Patients with hepatocellular carcinoma, fever, human immunodeficiency virus infection, overt infectious disease (septicemia, pneumonia, urinary tract infection), renal insufficiency, or under immunomodulatory therapy were also excluded. Clinical profiles of the patients are presented in Table 1. Twelve healthy volunteers were recruited after matching for age, gender, and body mass index (BMI) with the patients and served as controls (eight men and four women, mean age 59 ± 8 y). The study protocol was approved by the institutional review board for human research, and informed consent was obtained from all participants. The study protocol was in agreement with the 1975 Helsinki Declaration as revised in 1983. We matched the patients and the controls not only by age and gender but also by BMI, because obesity is reported to affect significantly the histologic grade of hepatic fat deposition, inflammation, and fibrosis even in

chronic viral hepatitis [24,25], thus reducing the functional reserve of the liver.

Methods

Blood was drawn in the early morning for fasting serum concentrations of TNF-α, sTNF-R55, sTNF-R75, leptin, and routine laboratory examinations on the day of metabolic studies. Serum albumin, total bilirubin, alanine aminotransferase, prothrombin activity, and urinary nitrogen (UN) were measured with a standard clinical analyzer at the central laboratory in our hospital. Serum TNF-α, sTNF-R55, and sTNF-R75 were determined in duplicate with an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA). Serum leptin was measured in duplicate by radioimmunoassay (Linco Research, Inc., St. Louis, MO, USA).

Metabolic studies were carried out using an indirect calorimeter (Deltatrac Metabolic Monitor, Datax Division Instrumentarium Corp., Helsinki, Finland) to estimate non-protein respiratory quotient (npRQ), resting energy expenditure (REE), and substrate oxidation rates of carbohydrate (%CHO), fat (%FAT), and protein (%PRO) from measured oxygen consumption per minute (Vo_2), carbon dioxide production per minute (Vco_2) and total UN using the following equations [26–28].

$$REE = 5.50\ Vo_2 + 1.76\ Vco_2 - 1.99\ UN$$

$$CHO(g/24\ h) = 5.926\ Vo_2 - 4.189\ Vco_2 - 2.539\ UN$$

$$FAT(g/24\ h) = 2.432\ Vo_2 - 2.432\ Vco_2 - 1.943\ UN$$

$$PRO(g/24\ h) = 6.250\ UN$$

$$npRQ = (1.44 \times Vco_2 - 4.890\ UN)/(1.44 \times Vo_2 - 6.04\ UN)$$

$$\%CHO = CHO \times 4.18/REE \times 100$$

$$\%FAT = FAT \times 9.46/REE \times 100$$

$$\%PRO = PRO \times 4.32/REE \times 100$$

Measurements were performed between 07:00 and 09:00 h while the patients were still lying in bed. The last meal was served 18:00 h on the previous day. The basal metabolic rate was predicted by the Harris-Benedict formula [29].

We measured height and body weight and calculated BMI. Arm circumference and TSF were measured with an Insertape and Adipometer (Abbott Japan Co., Ltd., Tokyo, Japan), and AMC was estimated. Anthropometric data were standardized according to age- and gender-stratified Japanese anthropometric reference data [30] and expressed as percentages of TSF and AMC.

Statistical analysis

Values were expressed as median and range. Comparisons between groups were analyzed using Mann-Whitney U or Kruskal-Wallis non-parametric test. Comparison between measured REE and corresponding Harris-Benedict prediction was performed by paired *t* test. The relation among blood test parameters and substrate oxidation rates or Child-Pugh grade was determined by Spearman's correlation.

All analyses were carried out using JMP 5.0 (SAS Institute, Cary, NC, USA) and statistical significance was accepted at $P < 0.05$.

Results

Serum concentrations of TNF-α, sTNF-R55, sTNF-R75, and leptin

Cirrhotic patients had significantly lower %TSF and %AMC in parallel with the increasing Child-Pugh score (Table 1), suggesting the presence of PEM in these subjects.

The median serum concentrations of TNF-α, sTNF-R55, and sTNF-R75 were significantly higher in cirrhotic patients than in controls (Table 1). Serum TNF-α, sTNF-R55, and sTNF-R75 levels correlated with the increasing grade of disease severity as defined by the Child-Pugh classification in patients with liver cirrhosis (Table 1, Fig. 1).

Serum leptin concentration did not differ between cirrhotic patients and controls or correlate with Child-Pugh score (Table 1), but correlated with their BMI (Fig. 2).

Energy metabolism

Measured REE (1188 kcal/d, 892–1830 kcal/d) was significantly higher than the basal metabolic rate (1170 kcal/d, 1077–1760 kcal/d) predicted by the Harris-Benedict formula in liver cirrhosis ($P < 0.01$), whereas both agreed well in control subjects.

The npRQ in patients with liver cirrhosis (0.84, 0.70–0.97) was significantly lower than that in control subjects (0.91, 0.87–0.94, $P < 0.01$). Decrease in npRQ was brought about by a significantly lower oxidation rate of carbohydrate and a higher oxidation rate of fat in patients with liver cirrhosis compared with control subjects (Fig. 3). A decrease in %CHO and an increase in %FAT significantly correlated with the progression of disease severity in patients with liver cirrhosis as defined by the Child-Pugh classification (Fig. 3).

Correlation between serum cytokine levels and oxidation rates of nutrients in patients with liver cirrhosis

Inverse correlations were found between %CHO and serum concentrations of TNF-α, sTNF-R55, and sTNF-R75 (Fig. 4, Table 2). Significant correlations were observed between %FAT and serum concentrations of TNF-α, sTNF-R55,

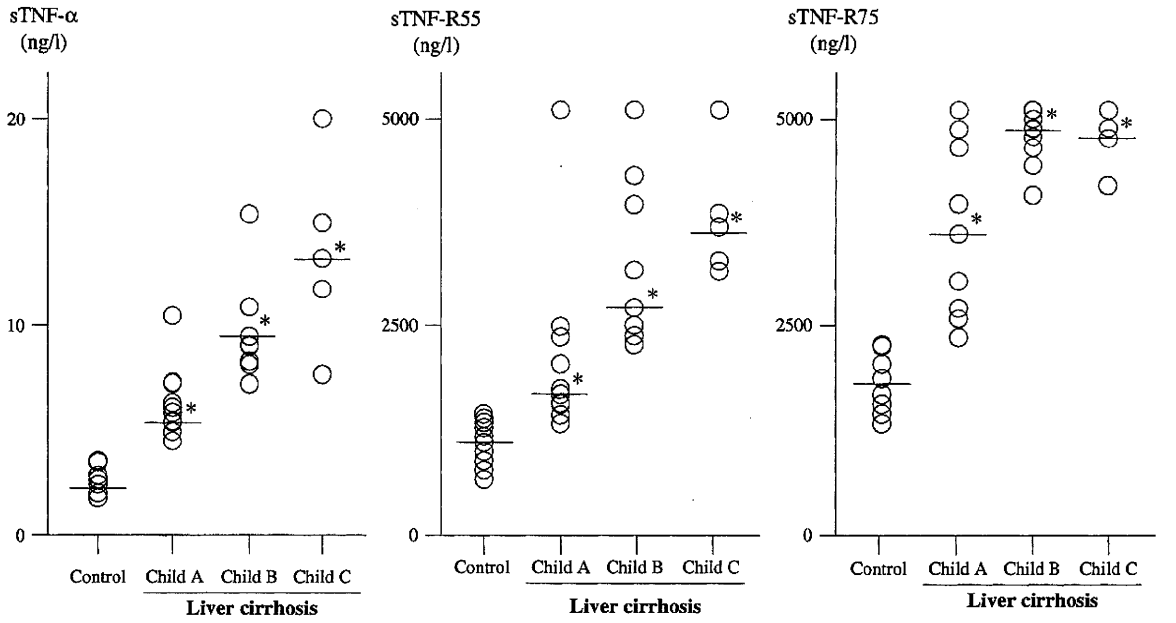

Fig. 1. Serum levels of TNF-α, sTNF-R55, and sTNF-R75 in control subjects ($n = 12$) and in patients with liver cirrhosis ($n = 24$) graded by Child-Pugh classification ($n = 9$, 9, and 6 for grades A, B, and C, respectively). Horizontal line indicates the median. * $P < 0.05$ compared with control. Child, Child-Pugh grade; sTNF-R55, soluble 55-kDa tumor necrosis factor receptor; sTNF-R75, soluble 75-kDa tumor necrosis factor receptor receptor; TNF-α, tumor necrosis factor-α.

and sTNF-R75 (Fig. 5, Table 2). However, %PRO did not correlate with serum concentrations of these cytokines.

Other intercorrelations among cytokines, leptin, substrate oxidation rates, and Child- Pugh grade in patients with liver cirrhosis are presented in Table 2. Serum leptin did not correlate with %CHO or %FAT. Serum leptin did not correlate with TNF-α, sTNF-R55, or sTNF-R75.

Correlations of serum cytokine and leptin levels with parameters of liver damage in cirrhotic patients

The serum concentrations of TNF-α, sTNF-R55, and sTNF-R75 correlated significantly with Child-Pugh score (Table 2), prothrombin time, total bilirubin, and albumin in patients with liver cirrhosis. However, serum leptin level showed no correlation with such parameters (data not shown).

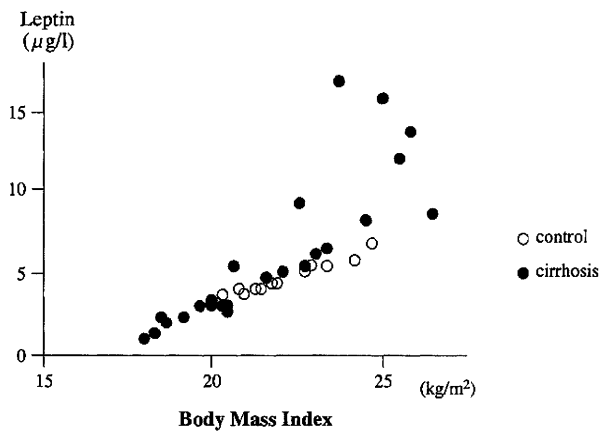

Fig. 2. Correlation between body mass index and serum leptin level in cirrhotic patients (closed circles) and controls (open circles; $n = 36$, $r = 0.735$, $P < 0.05$).

Discussion

Because the liver plays a central role in fuel and energy metabolism, PEM is common in patients with liver cirrhosis [1,2]. Recently, several studies have elucidated the relation between PEM and event-free survival in patients with liver cirrhosis [3–5,31,32]. Thus, PEM is an important outcome marker and a therapeutic target in cirrhotic patients.

It has been reported that the fasting oxidation rate of glucose is decreased and that of fat is increased in patients with liver cirrhosis [5,6,26]. Such aberrant energy metabolism consequently decreases npRQ in cirrhotics [5,6,26]. It also has been reported that this decrease of npRQ closely correlates with survival in patients with liver cirrhosis [5]. As confirmed in this study, npRQ in liver cirrhosis was significantly lower than that in control subjects. The decrease in npRQ was brought about by a significantly lower oxidation rate of carbohydrate and higher oxidation rate of fat in patients with liver cirrhosis compared with control subjects. A decrease in %CHO and an increase in %FAT significantly correlated with the progression of disease severity in patients with liver cirrhosis as defined by the Child-Pugh classification. These characteristics of energy metabolism in liver cirrhosis agree well with those in previous reports [5,6,26], suggesting that the patients recruited in the present study can be regarded as representative of a general cirrhotic cohort. Regarding

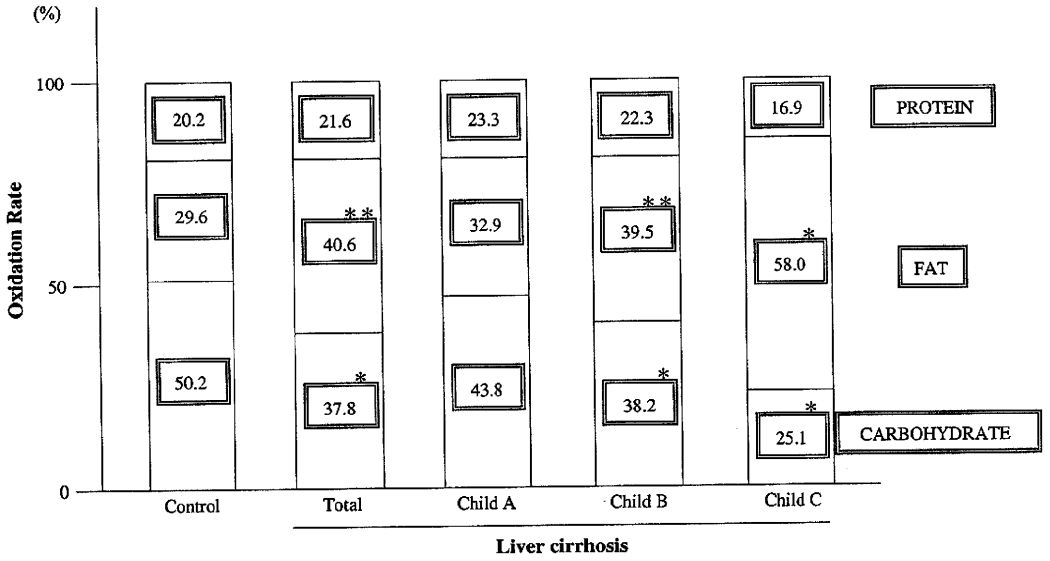

Fig. 3. Substrate oxidation rates of protein, fat, and carbohydrate in control subjects ($n = 12$) and in patients with liver cirrhosis ($n = 24$) graded by Child-Pugh classification ($n = 9$, 9, and 6 for grades A, B, and C, respectively). Values are expressed as mean. * $P < 0.05$, ** $P < 0.01$ compared with control. Child, Child-Pugh grade.

%TSF as another parameter of energy nutrition, the patients actually showed a significantly lower value than the controls with a similar BMI. However, we should point out the possibility that the patients could have had an overestimated BMI due to the presence of excess fluid before the appearance of detectable ascites or moderate to severe peripheral edema.

Tumor necrosis factor-α is a proinflammatory cytokine and postulated to regulate energy metabolism [9,10,33]. In previous reports, TNF-α increased glucose (~10%) and free fatty acid (~126%) turnover and raised REE (~34%) [9,10,33]. The actions of TNF-α are mediated by two distinct cell-surface receptors. We measured not only serum TNF-α concentration but also sTNF-R55 and sTNF-R75 concentrations for a couple of reasons. First, it is known that sTNF-R shedding is induced by the same stimuli that activate TNF-α production. Second, it has been shown that sTNF-R levels remain elevated for a longer time than TNF-α itself. Taken together, sTNF-R levels could confirm the stimulated state of the TNF-α system and could reflect the state until later after stimuli.

In this study, serum TNF-α, sTNF-R55, and sTNF-R75 concentrations were significantly higher in cirrhotic patients

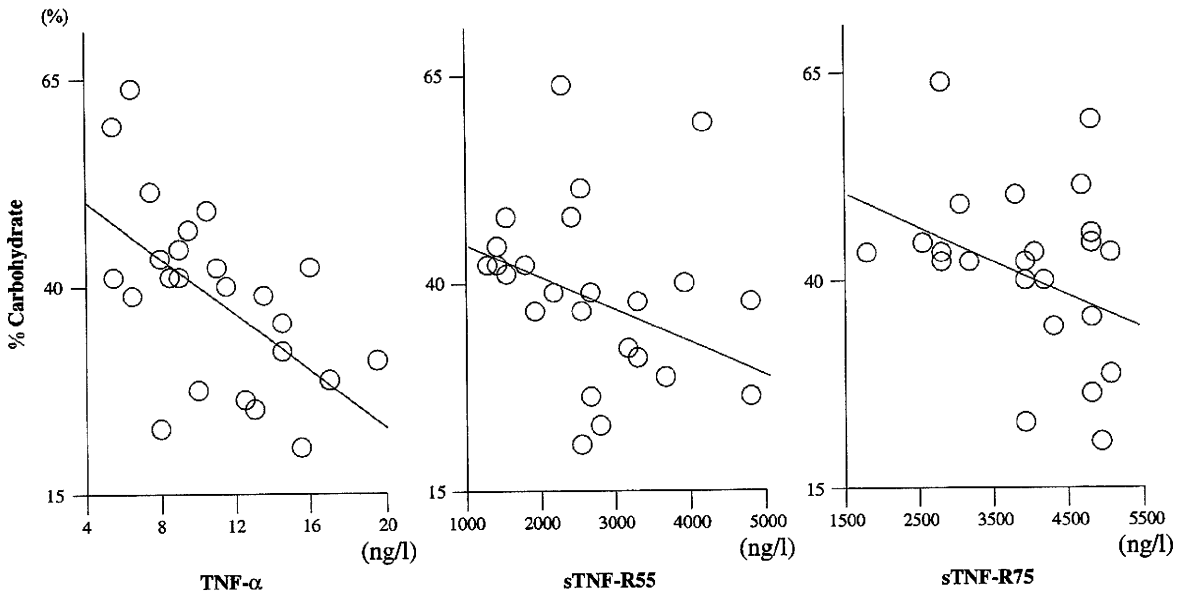

Fig. 4. Correlation between the oxidation rate of carbohydrate and serum TNF-α, sTNF-R55, or sTNF-R75 in patients with liver cirrhosis ($n = 24$). For correlation coefficients and statistical significance, see Table 2. sTNF-R55, soluble 55-kDa tumor necrosis factor receptor; sTNF-R75, soluble 75-kDa tumor necrosis factor receptor receptor; TNF-α, tumor necrosis factor-α.

Table 2
Intercorrelation coefficients among cytokines, leptin, substrate oxidation rates, and Child-Pugh score in patients with liver cirrhosis

	TNF-α	sTNF-R55	sTNF-R75	%CHO	%FAT	Leptin
sTNF-R55	0.32					
sTNF-R75	0.43*	0.85†				
%CHO	−0.56†	−0.44*	−0.41*			
%FAT	0.72†	0.61†	0.61†	−0.82†		
Leptin	−0.15	−0.01	0.06	0.08	−0.02	
Child-Pugh score	0.63†	0.61†	0.61†	−0.64†	0.78†	−0.36

%CHO, substrate oxidation rate of carbohydrate; %FAT, substrate oxidation rate of fat; TNF-α, tumor necrosis factor-α; sTNF-R55, soluble 55-kDa tumor necrosis factor receptor; sTNF-R75, soluble 75-kDa tumor necrosis factor receptor.

* $P < 0.05$.

† $P < 0.01$.

than in controls, and they correlated with the increasing grade of disease severity as defined by the Child-Pugh classification in patients with liver cirrhosis. Among damaged liver functions in cirrhosis, the high activity of the TNF-α system may particularly be related to a decrease in hepatic clearance of endotoxins, the presence of portosystemic shunts, and the systemic spillover of intestinal endotoxins by portal hypertension [15,34]. Another explanation is that the presence of chronic viral inflammation could be the reason for the elevated proinflammatory cytokines including TNF-α [35,36].

Moreover, inverse correlations were found between %CHO and serum concentrations of TNF-α, sTNF-R55, and sTNF-R75 in this study. Significant correlations were observed between %FAT and serum concentrations of TNF-α, sTNF-R55, and sTNF-R75. As described previously, TNF-α induces free fatty acid oxidation more efficiently than glucose oxidation [6,9,10]. In addition, the production of adenosine triphosphate by β-oxidation of free fatty acids is much greater than that by glucose oxidation. Hence, TNF-α seems to raise %FAT more directly and to contribute to whole aberrant energy metabolism subsequently in liver cirrhosis by altering the proportion of fat oxidation to carbohydrate oxidation.

Serum leptin is another candidate factor to alter energy balance by its wasting action and is reported to be actually high in patients with liver cirrhosis [11,20–22]. However, in this study, serum leptin concentration did not differ between cirrhotic patients and controls. Moreover, serum leptin level showed no correlation with energy metabolism parameters measured by indirect calorimetry. Most previous studies regarding leptin recruited patients with alcoholic cirrhosis [20,22], whereas all patients in our investigation had cirrhosis caused by hepatitis C virus. This difference in the cause of liver damage may account for the disagreement between previous reports and the present one.

Regarding the correlation between TNF and aberrant energy metabolism, we should point out a possibility that the correlation may merely arise from an association between cytokine levels and Child-Pugh grade, as shown in Figure 1, and that between substrate oxidation rates and the grade, as presented in Table 2. To support our hypothesis that the correlation between TNF and energy metabolism is independent of the degree of liver damage, we statistically tested the relation by confining subjects to those with each Child-Pugh grade. In each subgroup that was controlled for grade, TNF and %FAT retained the significant correlation (e.g., $r = 0.81$, $P < 0.01$ in Child-Pugh grade A). These results strengthen our statement

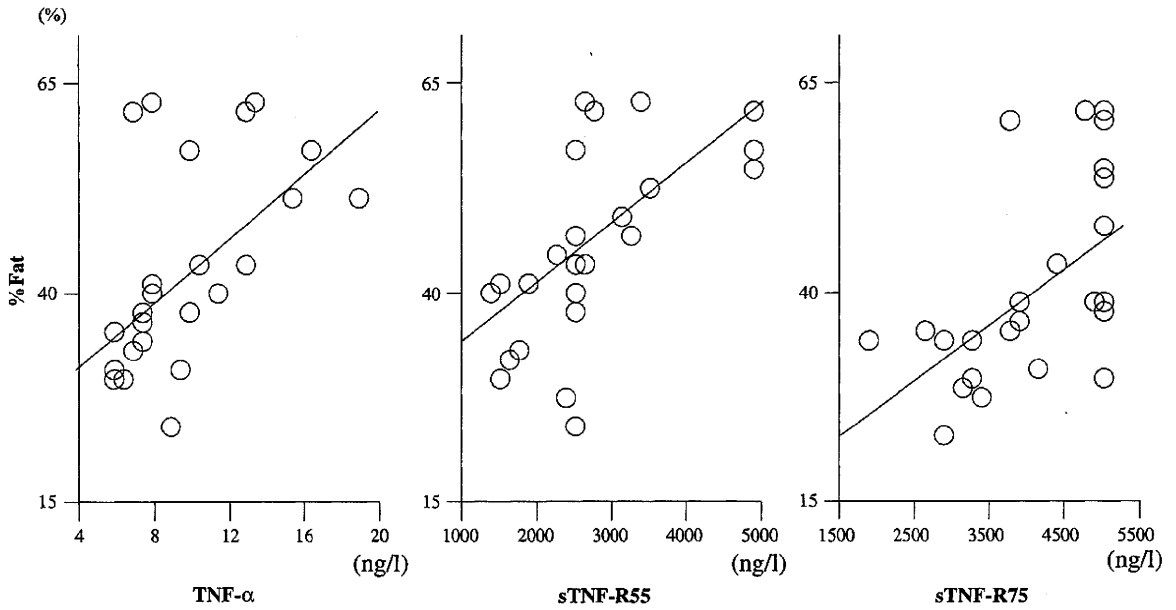

Fig. 5. Correlation between the oxidation rate of fat and serum TNF-α, sTNF-R55, or sTNF-R75 in patients with liver cirrhosis ($n = 24$). For correlation coefficients and statistical significance, see Table 2. sTNF-R55, soluble 55-kDa tumor necrosis factor receptor; sTNF-R75, soluble 75-kDa tumor necrosis factor receptor receptor; TNF-α, tumor necrosis factor-α.

that TNF-α might be associated with aberrant energy metabolism in patients with liver cirrhosis.

Taken together, it seems more likely that the high activity of the TNF-α system mediates the aberrant energy metabolism in patients with liver cirrhosis. To directly demonstrate this possibility, future clinical trials would be required to test whether or not the therapy to decrease the high TNF-α activity might correct the aberrant energy metabolism in patients with liver cirrhosis, by using such modalities as antibiotics and lactulose that control the intestinal bacterial flora or anti–TNF-α antibody.

In summary, the TNF-α system in patients with liver cirrhosis was activated and correlated with disease activity. The significant correlation of serum TNF-α, sTNF-R55, and sTNF-R75 with the aberrant energy metabolism suggested that activation of the TNF-α system contributed to cirrhosis-associated PEM.

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Dietary supplementation with branched-chain amino acids suppresses diethylnitrosamine-induced liver tumorigenesis in obese and diabetic C57BL/KsJ-*db/db* mice

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Obesity and related metabolic abnormalities, including insulin resistance, are risk factors for hepatocellular carcinoma in non-alcoholic steatohepatitis as well as in chronic viral hepatitis. Branched-chain amino acids (BCAA), which improve insulin resistance, inhibited obesity-related colon carcinogenesis in a rodent model, and also reduced the incidence of hepatocellular carcinoma in obese patients with liver cirrhosis. In the present study, we determined the effects of BCAA on the development of diethylnitrosamine (DEN)-induced liver tumorigenesis in obese C57BL/KsJ-*db/db* (*db/db*) mice with diabetes mellitus. Male *db/db* mice were given tap water containing 40 ppm DEN for an initial 2 weeks and thereafter they received a basal diet containing 3.0% of BCAA or casein, which served as a nitrogen content-matched control of BCAA, throughout the experiment. Supplementation with BCAA significantly reduced the total number of foci of cellular alteration, a premalignant lesion of the liver, and the expression of insulin-like growth factor (IGF)-1, IGF-2, and IGF-1 receptor in the liver when compared to the casein supplementation. BCAA supplementation for 34 weeks also significantly inhibited both the development of hepatocellular neoplasms and the proliferation of hepatocytes in comparison to the basal diet or casein-fed groups. Supplementation with BCAA improved liver steatosis and fibrosis and inhibited the expression of α-smooth muscle actin in the DEN-treated *db/db* mice. The serum levels of glucose and leptin decreased by dietary BCAA, whereas the value of the quantitative insulin sensitivity check index increased by this agent, indicating the improvement of insulin resistance and hyperleptinemia. In conclusion, oral BCAA supplementation improves insulin resistance and prevents the development of liver tumorigenesis in obese and diabetic mice. (*Cancer Sci* 2010; 101: 460–467)

Hepatocellular carcinoma is a major health problem worldwide. The development of HCC is frequently associated with chronic inflammation of the liver induced by a persistent infection with the hepatitis B virus or hepatitis C virus.[1] The risk of HCC is also elevated in those with metabolic syndrome, also called insulin resistance syndrome, which is commonly associated with obesity and impaired glucose tolerance.[1–4] Non-alcoholic fatty liver disease is known to be a hepatic manifestation of the metabolic syndrome. Diabetes mellitus, a condition associated with hyperinsulinemia, has been proposed as a risk factor for both chronic liver disease and HCC through the development of NASH, which is observed in a subset of patients with non-alcoholic fatty liver disease and involves inflammation, cell damage, and/or fibrosis in the liver.[5–7] In 1998, Day and James proposed, in their "two hit theory," that insulin resistance is regarded as a critical factor in the etiology of NASH.[8]

C57BL/KsJ-*db/db* (*db/db*) mice are a genetically altered animal model with phenotypes of obesity and diabetes mellitus. A functional defect in the long-form leptin receptor, which plays a significant role in the regulation of food intake and the control of body weight, leads to hyperleptinemia in these mice.[9] Because of such obesity, hyperinsulinemia, and hyperleptinemia, the *db/db* mice represent a suitable animal model that uniquely mimics the metabolic syndrome in humans.[10] It is also reported that the *db/db* mice are susceptible to chemically induced carcinogenesis in certain tissues. For instance, the *db/db* mice are sensitive to AOM-induced colon carcinogenesis; putative precursor lesions for colorectal cancer are greatly enhanced in *db/db* mice compared to *db/+* or *+/+* mice.[11] With respect to the liver, feeding *db/db* mice with a methionine and choline-deficient diet developed accelerated hepatic inflammation and fibrosis, very similar to those seen in human NASH.[12]

An improvement of insulin resistance by nutritional or pharmaceutical intervention might therefore be an effective and attractive strategy to inhibit the obesity-related carcinogenesis, as already reported experimentally for the colon.[13] Candidate modalities include dietary supplementation with BCAA (leucine, isoleucine, and valine) because BCAA prevents progressive hepatic failure and improves the event-free survival in patients with chronic liver diseases, at least in part, by improving insulin resistance.[14–16] In addition, BCAA supplementation has been shown to prevent obesity-related colon carcinogenesis initiated with AOM[17] and, furthermore, to reduce the risk of HCC in obese patients with chronic viral liver disease.[18] In an obesity-related colon cancer model, the effects of BCAA in inhibiting the development of colonic premalignancies might be associated with improvement of insulin resistance.[17] However, whether BCAA prevents obesity-related liver carcinogenesis, and the precise mechanisms of that prevention, have not been explored.

In the present study, we examined the effects of BCAA supplementation on the development of HCC, liver cell adenoma, and FCA in obese and diabetic *db/db* mice initiated with DEN by focusing on the improvement of insulin resistance, liver steatosis, and fibrosis. We also examined whether BCAA supplementation in the diet alters the expression of IGF-1, IGF-2, and IGF-1R in the liver of DEN-treated *db/db* mice. The IGF/IGF-1R axis is closely associated with the development of HCC and might be regarded as a critical target for both HCC treatment and chemoprevention.[19,20]

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Materials and Methods

Animals, chemicals, and diets. Four-week-old male *db/db* mice were obtained from Japan SLC (Shizuoka, Japan). All mice received humane care and were maintained at Gifu University Life Science Research Center (Gifu, Japan), according to the Institutional Animal Care Guidelines. DEN was purchased from Sigma Chemical Co. (St. Louis, MO, USA). BCAA and casein were obtained from Ajinomoto Co. (Tokyo, Japan). The BCAA composition (2:1:1.2 = leucine:isoleucine:valine) was set at the clinical dosage that is used for the treatment of decompensated liver cirrhosis in Japan.[16,18] The basal diet, CRF-1 (Oriental Yeast Co., Tokyo, Japan), contained 22.4 g of protein (1.65 g leucine, 0.83 g isoleucine, and 1.03 g valine) per 100 g of total volume.

Experimental procedure. The experimental protocol was approved by the Institutional Committee of Animal Experiments of Gifu University. At 5 weeks of age, a total of 41 *db/db* mice were divided into three groups. All the mice in Group 1 ($n = 11$), Group 2 ($n = 15$), and Group 3 ($n = 15$) were given tap water containing 40 ppm DEN for the initial 2 weeks. After treatment with DEN, Group 3 was given the CRF-1 supplemented with 3.0% BCAA (w/w) through to the end of experiment, whereas mice in Group 2 were given the basal diet supplemented with 3.0% casein (w/w) and served as a nitrogen content-matched control for the BCAA-treated group. Group 1 was given the CRF-1 diet throughout the experiment. In order to examine the effect of BCAA on the development of FCA in early phase, four mice each in groups 2 and 3 were starved for 6 h and killed by CO_2 asphyxiation at 23 weeks of age (after 16 weeks supplementation with BCAA or casein). At 41 weeks of age (after 34 weeks supplementation with the experimental diet), all remaining animals (total 33 mice) were killed to determine the development of HCC, liver cell adenoma, and FCA.

Histopathology and immunohistochemical analyses for α-SMA and PCNA. After the mice were killed, the livers were immediately removed and macroscopically inspected for the presence of neoplasms. Maximum sagittal sections of each lobe (six lobes) were used for histological examination. The tissue specimens were fixed in 10% buffered formaldehyde then embedded in paraffin. Serial sections (3–4 μm thick) were cut from the tissue blocks and stained with H&E for histopathology or Azan stain to observe liver fibrosis. The liver neoplasms (HCC and liver cell adenoma) and FCA were diagnosed according to criteria described previously.[21] The multiplicity of FCA was assessed on the per area basis (per cm^2).

Immunohistochemistry of α-SMA, an indicator of HSC activation, was carried out using a primary anti-α-SMA antibody (Dako, Glostrup, Denmark).[22] Immunohistochemistry of PCNA, a G_1-to-S phase marker, was carried out to estimate the cell proliferative activity of the hepatocyte using a primary anti-PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).[23] PCNA-positive nuclei in the hepatocytes were counted and expressed as the percentage of the total number of hepatocyte nuclei. The PCNA-labeling index (%) was determined by counting at least 500 hepatocytes in each section (total of 3000 hepatocytes per mouse).

Clinical chemistry. After the mice were killed, blood samples were collected from inferior vena cava to determine the serum concentrations of ALT, glucose, insulin, leptin, and BCAA. The levels of serum glucose, insulin, and BCAA were assayed as described previously.[24,25] The serum leptin level was determined by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.[17] The serum ALT activity was measured with a standard clinical automatic analyzer (type 726; Hitachi, Tokyo, Japan). Insulin resistance was estimated by QUICKI as follows: $QUICKI = 1/[\log(I_0) + \log(G_0)]$, where I_0 is the fasting insulin and G_0 is the fasting glucose, which correlates with the glucose clamp method.[26]

Hepatic lipid analysis. To visualize intrahepatic lipids, Sudan III stain was carried out with frozen sections using the standard procedure. The hepatic lipids were also extracted from the frozen livers. Approximately 200 mg of liver was homogenized and the lipids were then extracted using chloroform:methanol (2:1 v/v) solution, as described by Folch.[27] The levels of triglyceride in the liver were measured using the triglyceride E-test kit (Wako Pure Chemical Co., Osaka, Japan) according to the manufacturer's protocol.

Hepatic hydroxyproline analysis. Hepatic hydroxyproline content was quantified colorimetrically in duplicate samples from approximately 200 mg wet-weight of liver tissue, as previously described.[22] The hydroxyproline contents were expressed as μmol/g wet liver.

Protein extraction and Western blot analysis. Equivalent amounts of protein lysates (30 μg/lane) from the liver of experimental mice were subjected to a Western blot analysis of α-SMA (Dako), as described previously.[22,23] An antibody to GAPDH (Chemicon International, Temecula, CA, USA) served as a loading control. The intensities of the blots were quantified with NIH image software version 1.62.

RNA extraction and quantitative real-time RT-PCR analysis. A quantitative real-time RT-PCR analysis was carried out as described previously.[28] Total RNA was isolated from the liver of the mice using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX, USA). The cDNA was synthesized from 0.2 μg total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The primers used for the amplification of *IGF-1*, *IGF-2*, and *IGF-1R* specific genes were as follows: *IGF-1* forward, 5′-CTGGACCAGAGACCCTTTGC-3′ and reverse, 5′-GGACGGGGACTTCTGAGTCTT-3′; *IGF-2* forward, 5′-GTGCTGCATCGCTGCTTAC-3′ and reverse, 5′-ACGTCCCTCTCGGACTTGG-3′; and *IGF-1R* forward, 5′-GTGGGGGCTCGTGTTTCTC-3′ and reverse, 5′-GATCACCGTGCAGTTTTCCA-3′. Real-time PCR was done in a LightCycler (Roche Diagnostics Co., Indianapolis, IN, USA) with SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan). The expression levels of the *IGF-1*, *IGF-2*, and *IGF-1R* genes were normalized to the β-*actin* gene expression level.[28]

Statistical analysis. The results are presented as the mean ± SD, and they were analyzed using the GraphPad Instat software program version 3.05 (GraphPad Software, San Diego, CA, USA) for Macintosh. Differences among the groups were analyzed by either one-way ANOVA or, as required, by two-way ANOVA. When ANOVA showed a statistically significant effect ($P < 0.05$), comparisons of each experimental group with the control group were then made using Dunnett's test, which corrects for multiple comparisons. The differences were considered to be significant when the two-sided P value was <0.05.

Results

General observations. As shown in Table 1, there were no significant differences in the body, liver, kidney, or fat (white adipose tissue of the periorchis and retroperitoneum) weights among the groups at the end of the study. Male *db/db* mice well-tolerated the treatment with DEN together with casein or BCAA. The body weight gains did not differ significantly among the groups during the experiment (data not shown). A histopathological examination suggested the absence of toxicity of BCAA in important organs, including liver, kidney, and spleen (data not shown). In addition, no clinical signs indicating the toxicity of BCAA were observed in the mice during the experiment.

Incidence and multiplicity of DEN-induced liver neoplasms and FCA in *db/db* mice. Macroscopically, nodular lesions (Fig. 1a) were observed in the livers of experimental mice at the termination of the study (41 weeks of age). Histopathologically, these lesions were liver cell adenoma (Fig. 1b) or HCC (Fig. 1c).

Table 1. Body, liver, kidney, spleen, and fat weights of the experimental mice

Group no.	Diet	No. of mice	Weight (g) (mean ± SD)			
			Body	Liver	Kidney	Fat†
1	CRF-1	11	73.0 ± 9.2	4.4 ± 0.9	0.9 ± 1.0	7.8 ± 2.2
2	Casein	11	66.0 ± 12.0	3.8 ± 1.2	0.6 ± 0.2	5.4 ± 1.0
3	BCAA	11	68.2 ± 12.4	3.4 ± 1.3	0.6 ± 0.1	6.2 ± 1.4

†White adipose tissue of the periorchis and retroperitoneum.

FCA (Fig. 1d) also developed in the liver of experimental mice. Simultaneously, we put supplemental groups to support that *db/db* mice are actually susceptible to DEN-induced liver tumorigenesis (data not shown; see Supporting Information Table S1) and found no neoplasms in C57B6 or C57BL/KsJ-+/+ mice, genetic controls for *db/db* mice, regardless of DEN treatment. No tumors developed in the CRF-1-fed and DEN-untreated *db/db* mice.

Effects of BCAA supplementation on DEN-induced liver tumor igenesis in *db/db* mice. The incidence and multiplicity of liver neoplasms (adenoma plus HCC) and FCA at 41 weeks of age are summarized in Table 2. Compared with the CRF-1-fed mice (Group 1), dietary supplementation with BCAA (Group 3) significantly inhibited the incidence ($P < 0.05$) of adenoma. BCAA supplementation also reduced the incidence ($P < 0.05$) and multiplicity of adenoma ($P < 0.01$) compared to the casein-supplementation mice (Group 2). HCC was developed in the CRF-1-fed (9%) and casein-supplementation mice (27%), but not in the mice supplemented with BCAA (0%), and the multiplicity of total liver neoplasms was significantly inhibited by supplementation with BCAA when compared to CRF-1-fed ($P < 0.05$) or Casein-supplementation mice ($P < 0.01$), respectively. The number of FCA, which were developed in all experimental mice, was also significantly decreased by supplementation with BCAA when compared to CRF-1-fed ($P < 0.05$) or casein-supplementation mice ($P < 0.001$), respectively.

Effects of BCAA supplementation on the expression levels of *IGF-1*, *IGF-2*, and *IGF-1R* mRNAs in the liver of DEN-treated *db/db* mice. When the mice were killed at 23 weeks of age, the development of FCA was also significantly inhibited by dietary supplementation with BCAA compared with casein-supplemented mice ($P < 0.01$) (Fig. 2a). In addition, semiquantitative RT-PCR analyses showed that there was a significant decrease in the expression level of *IGF-1* ($P < 0.05$), *IGF-2* ($P < 0.05$), and *IGF-1R* mRNAs ($P < 0.05$) in the livers of the mice supplemented with BCAA when compared to that of the livers in casein-supplemented mice (Figs 2b–d). These findings suggest that BCAA supplementation prevents the development of FCA, at least in part, by inhibiting the expression of the IGF/IGF-1R axis.

Effects of BCAA supplementation on serum levels of BCAA, ALT, and leptin in DEN-treated *db/db* mice. BCAA supplementation caused a significant increase in the serum levels of BCAA compared to the CRF-1-fed ($P < 0.05$) and casein-supplemented mice ($P < 0.05$) (Fig. 3a). These findings suggest that supplementation with 3.0% BCAA is sufficient to raise the serum concentration of BCAA. The serum ALT levels markedly increased in the *db/db* mice when compared to the genetic control mice (data not shown; see Supporting Information Table S1). However, BCAA supplementation significantly decreased this value in comparison to the CRF-1-fed ($P < 0.01$) and casein-supplemented mice ($P < 0.001$) (Fig. 3b), thus indicating an improvement of liver damage. In addition, the mice supplemented with BCAA showed a decrease in the serum levels of leptin compared with the CRF-1-fed ($P < 0.001$) and casein-supplemented mice ($P < 0.001$) (Fig. 3c).

Effects of BCAA supplementation on the hepatic steatosis in DEN-treated *db/db* mice. Examination of Sudan III stained sections revealed that there was a marked macrovesicular steatosis in the DEN-treated *db/db* mice, which were fed CRF-1 or casein, but BCAA supplementation significantly improved the accumulation of the lipid in the liver (Fig. 4a). The histological findings were consistent with the results of the measurement of liver triglyceride contents; the levels of triglyceride in the liver of DEN-treated *db/db* mice were significantly decreased by the supplementation with BCAA compared to those in the CRF-1-fed ($P < 0.001$) and casein-supplementation groups ($P < 0.01$) (Fig. 4b).

Effects of BCAA supplementation on liver fibrosis in DEN-treated *db/db* mice. As shown in Figure 5a, examination of Azan-stained sections indicated that DEN-treated *db/db* mice of CRF-1-fed and casein-supplemented groups showed the development of peri-central venous and peri-cellular fibrosis. However, supplementation with BCAA yielded an improvement in liver fibrosis (Fig. 5a). Similar findings were also observed in

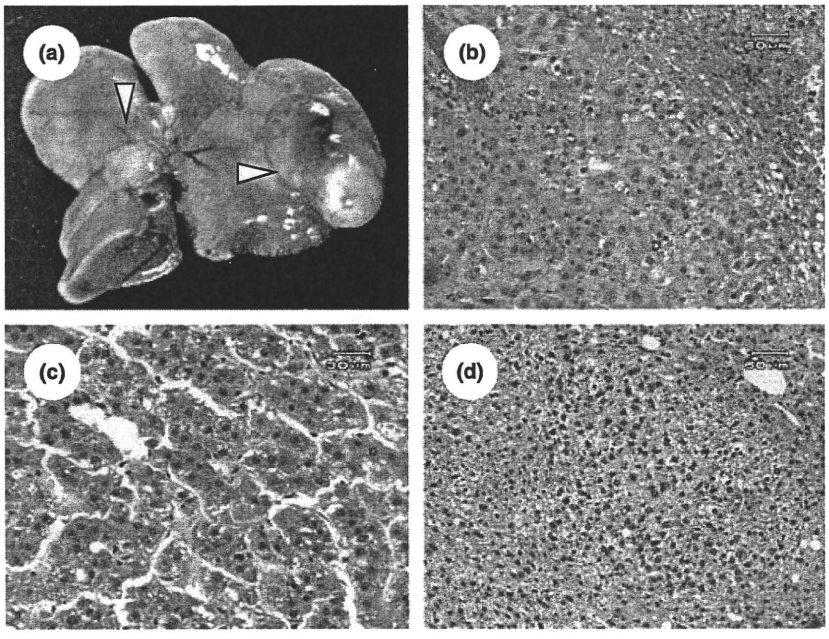

Fig. 1. Macroscopic (a) and microscopic (b–d) analyses of liver neoplasms in diethylnitrosamine-treated *db/db* mice. (a) Macroscopically, white tumors (hepatocellular carcinoma; indicated by arrowheads) were detected in the liver of diethylnitrosamine-treated C57BL/KsJ-*db/db* mice. (b–d) Paraffin-embedded sections were stained with H&E. Representative photomicrographs show adenoma (b), hepatocellular carcinoma (c), and foci of cellular alteration (d) in liver of experimental mice.

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Table 2. Incidence and multiplicity of hepatic neoplasms and foci of cellular alteration (FCA) in obese diabetic C57BL/KsJ-*db/db* mice fed basal (CRF-1), casein-supplemented, or branched-chain amino acid (BCAA)-supplemented diets

Group no.	Diet	No. of mice	Incidence (%)		Multiplicity (no. of neoplasms/mouse) (mean ± SD)			FCA (No./cm^2) (mean ± SD)
			Adenoma	HCC	Total	Adenoma	HCC	
1	CRF-1	11	7/11 (64)	1/11 (9)	1.0 ± 1.1	0.9 ± 1.1	0.1 ± 0.3	14.4 ± 4.4
2	Casein	11	8/11 (73)	3/11 (27)	1.7 ± 1.3	1.5 ± 1.1	0.3 ± 0.5	19.1 ± 5.7
3	BCAA	11	2/11 (18)*,**	0/11 (0)	0.2 ± 0.4*,***	0.2 ± 0.4***	0	9.6 ± 5.1*,****

*P < 0.05, significantly different from Group 1; **P < 0.05, significantly different from Group 2; ***P < 0.01, significantly different from Group 2; ****P < 0.001, significantly different from Group 2. HCC, hepatocellular carcinoma.

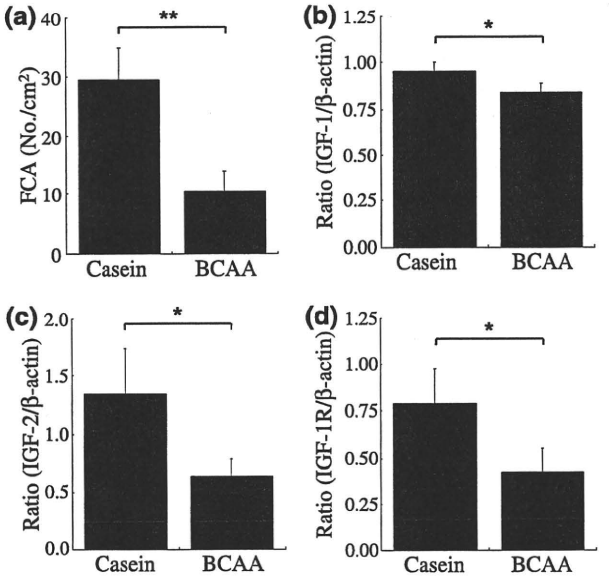

Fig. 2. Effect of branched-chain amino acid (BCAA) supplementation on the development of foci of cellular alteration (FCA) and on the expression of insulin-like growth factor (IGF)-1, IGF-2 and IGF-1 receptor (IGF-1R) mRNAs in the liver of diethylnitrosamine-treated C57BL/KsJ-*db/db* mice. Livers were excised from treated mice supplemented with casein or BCAA for 16 weeks. (a) Paraffin-embedded liver sections were stained with H&E and the total numbers of FCA were counted. Values are the means ± SD (n = 4). (b–d) Total RNA was isolated from the removed liver and the expression of *IGF-1* (b), *IGF-2* (c), and *IGF-1R* (d) genes were examined by quantitative real-time RT-PCR. The expression of each gene was normalized to β-actin expression. Each experiment was done in triplicate. *P < 0.05; **P < 0.01.

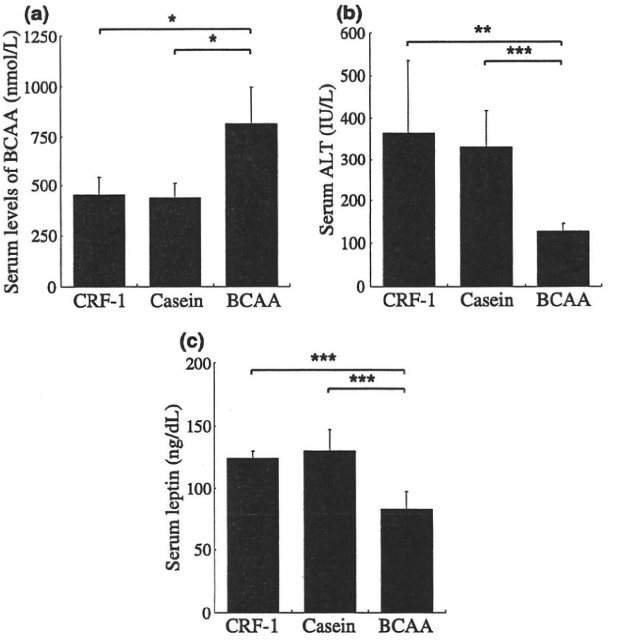

Fig. 3. Effect of branched-chain amino acid (BCAA) supplementation on the serum levels of BCAA, alanine aminotransferase (ALT), and leptin in diethylnitrosamine-treated C57BL/KsJ-*db/db* mice. After mice were killed, blood samples were collected and the serum levels of BCAA (a), ALT (b), and leptin (c) were then assayed. Values are the means ± SD (n = 8). *P < 0.05; **P < 0.01; ***P < 0.001.

the measurement of liver hydroxyproline contents, a useful marker of hepatic fibrosis;[22] when compared to CRF-1 feeding (P < 0.05) and casein supplementation (P < 0.01), BCAA supplementation caused a significant decrease in the amounts of hydroxyproline in the liver of DEN-treated *db/db* mice (Fig. 5b). In addition, both the immunohistochemical (Fig. 6a) and Western blot analyses (Fig. 6b) showed the expression levels of α-SMA in the liver to be elevated in the CRF-1-fed and casein-supplemented mice, whereas supplementation with BCAA significantly decreased the expression of this protein (P < 0.05 and P < 0.01, respectively).

Effects of BCAA supplementation on insulin resistance and serum level of glucose in DEN-treated *db/dbdb* mice. Insulin resistance plays a critical role in obesity-related HCC development.[1–4] Therefore, the effects of BCAA supplementation on the value of QUICKI and the serum levels of glucose were examined in DEN-treated *db/db* mice. As shown in Figure 7a, supplementation with BCAA caused a significant increase in the value of QUICKI compared to the CRF-1-fed (P < 0.01) and casein-supplemented mice (P < 0.01), thus indicating an improvement of insulin resistance. The serum glucose level also decreased after the supplementation with BCAA compared to CRF-1-fed (P < 0.001) and casein-supplementation (P < 0.01) (Fig. 7b).

Effects of BCAA supplementation on cell proliferative activity in liver of DEN-treated *db/db* mice. The PCNA-labeling index of non-lesional hepatocytes in DEN-treated *db/db* mice was determined based on the findings of PCNA-immunohistochemical sections (Fig. 8a). As illustrated in Figure 8b, the mean PCNA-labeling index in the BCAA-supplemented mice was significantly lower than that of the CRF-1-fed (P < 0.01) and casein-supplemented mice (P < 0.05), thus indicating that BCAA supplementation significantly inhibited cell proliferation in the liver of DEN-treated *db/db* mice.

Discussion

Recent studies have shown that obesity and diabetes mellitus are risk factors for HCC through the development of NASH.[5–7] The

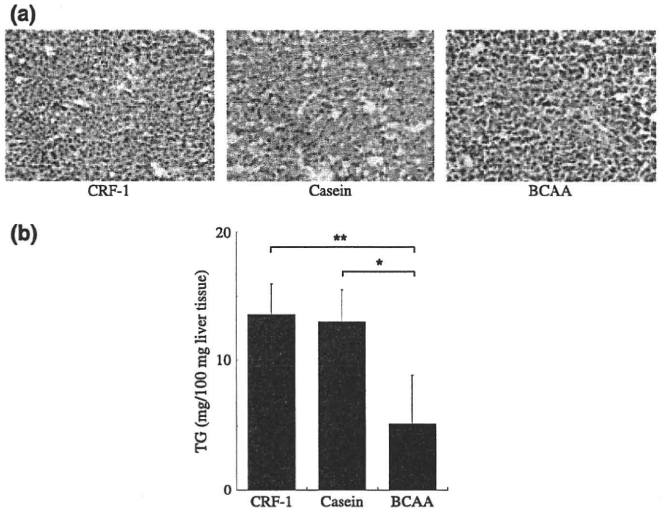

Fig. 4. Effect of branched-chain amino acid (BCAA) supplementation on hepatic steatosis in diethylnitrosamine-treated C57BL/KsJ-*db/db* mice. (a) Frozen sections of basal diet (CRF-1)-fed, casein-supplemented, or BCAA-supplemented treated mice were stained with Sudan III stain to show steatosis. (b) Hepatic lipids were extracted from the frozen livers and the levels of triglyceride were then measured. Values are the means ± SD (n = 8). *P < 0.01; **P < 0.001.

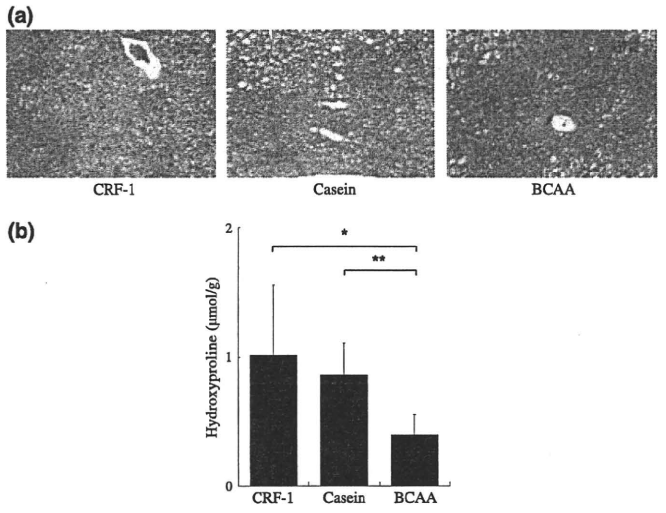

Fig. 5. Effect of branched-chain amino acid (BCAA) supplementation on hepatic fibrosis in diethylnitrosamine-treated C57BL/KsJ-*db/db* mice. (a) Paraffin-embedded sections of basal diet (CRF-1)-fed, casein-supplemented, or BCAA-supplemented treated mice were stained with Azan stain to show fibrosis. (b) Hepatic hydroxyproline contents were quantified colorimetrically. Values are the means ± SD (n = 8). *P < 0.05; **P < 0.01.

present study clearly indicated that *db/db* mice, which develop obesity and severe diabetes mellitus, easily developed steatosis-related liver neoplasms by treatment with liver carcinogen DEN (Table 2 and Fig. 1), whereas background C57B6 or C57BL/KsJ-+/+ mice did not. Furthermore, this study showed that dietary supplementation with BCAA effectively decreased the serum levels of ALT (Fig. 3b), which increase due to severe steatosis (Fig. 4a) and fibrosis (Fig. 5a), and inhibited the development of liver neoplasms (Table 2) in DEN-treated *db/db* mice. A clinical trial recently indicated that dietary supplementation with BCAA can reduce the risk of HCC in cirrhotic patients who are obese.[18] How can BCAA exert chemopreventive effects on obesity-related HCC? Presumably, the improvement of insulin resistance by BCAA (Fig. 7a) plays a critical role in this beneficial effect because, in addition to the role of insulin in glucose uptake and glycogen biosynthesis in liver and skeletal muscle, insulin has oncogenic properties on HCC cells, including the stimulation of cell growth and induction of anti-apoptotic activity.[29,30] These reports, therefore, suggest the possibility that BCAA inhibits the excessive cell proliferation in the whole liver of DEN-treated *db/db* mice (Fig. 8) by improving insulin resistance (Fig. 7a). Recent studies have also revealed that BCAA improves glucose tolerance by modulating the insulin-independent glucose uptake into skeletal muscle.[31,32] Isoleucine increased muscle glucose uptake and depressed gluconeogenesis in the liver without causing significant elevation of the plasma insulin level, thereby leading to the hypoglycemic effect in a rodent model.[33] Both improved insulin resistance and glucose tolerance by BCAA have also been indicated in clinical trials.[14,34]

In addition to the improvement of insulin resistance (Fig. 7a), the present study also indicated that dietary supplementation with BCAA significantly decreased the expression levels of *IGF-1*, *IGF-2*, and *IGF-1R* mRNAs in the liver of DEN-treated *db/db* mice (Figs 2b–d). These findings seem to be significant because abnormal activation of the IGF/IGF-1R axis, which is caused by insulin resistance, is involved in the development of HCC and, therefore, might be a critical target to prevent this malignancy.[19,20] These findings are also consistent with those of a previous report that showed BCAA supplementation decreased the serum levels of both IGF-1 and IGF-2 while also inhibiting the expression of IGF-1R on the colonic mucosa, thereby preventing the development of AOM-induced colonic neoplastic lesions in *db/db* mice.[17] This previous report,[17] together with our present findings (Fig. 2), suggest the possibility that the inhibition of IGF/IGF-1R activation is one of the critical mechanisms to suppress obesity-related tumorigenesis in specific organs, such as the colon and liver, and BCAA might be able to exert its chemopreventive effect on obesity-associated carcinogenesis by targeting this axis.

Insulin stimulates glucose uptake and triglyceride biosynthesis, which are stored in adipose tissue. The improvement of insulin resistance by BCAA, therefore, inhibits the release of free fatty acid from adipose tissue, improves hypertriglyceridemia, and thus resulted in improvement of hepatic steatosis in the present study (Fig. 4). Ectopic triglyceride accumulation in the liver is directly responsible for the development of insulin resistance.[35] In addition, several studies support the concept that hepatic steatosis promotes the development of HCC.[36] For instance, HCV core protein gene transgenic mice, a model for HCV-related hepatocarcinogenesis,[37] show marked hepatic steatosis and insulin resistance.[38,39] Hepatic steatosis is a major accelerating factor of hepatocarcinogenesis in chronic HCV infected patients.[40] In addition, a significant relationship has also been reported between steatosis and hepatic fibrosis, a potent risk factor for HCC development.[36] Therefore, the reduction of hepatic lipid accumulation might be an effective strategy for HCC chemoprevention. The improvement of hepatic steatosis (Fig. 4) and fibrosis (Fig. 5) by BCAA is thus considered to be advantageous to accomplish this objective.

There are two study limitations that might suggest additional investigations. The first is that the incidence of HCC itself was not very high in the present study (Table 2) because the duration of the experiments (41 weeks) might have been sufficient to develop adenoma but not HCC. Therefore, future study should recruit longer-term experiments to see that DEN-treated *db/db* mice develop HCC more frequently. The second is that, although our model seems to be useful to elucidate the pathogenesis underlying NASH-associated HCC, there is one difference between the liver of *db/db* mice and human NASH, as hepatic fibrosis was

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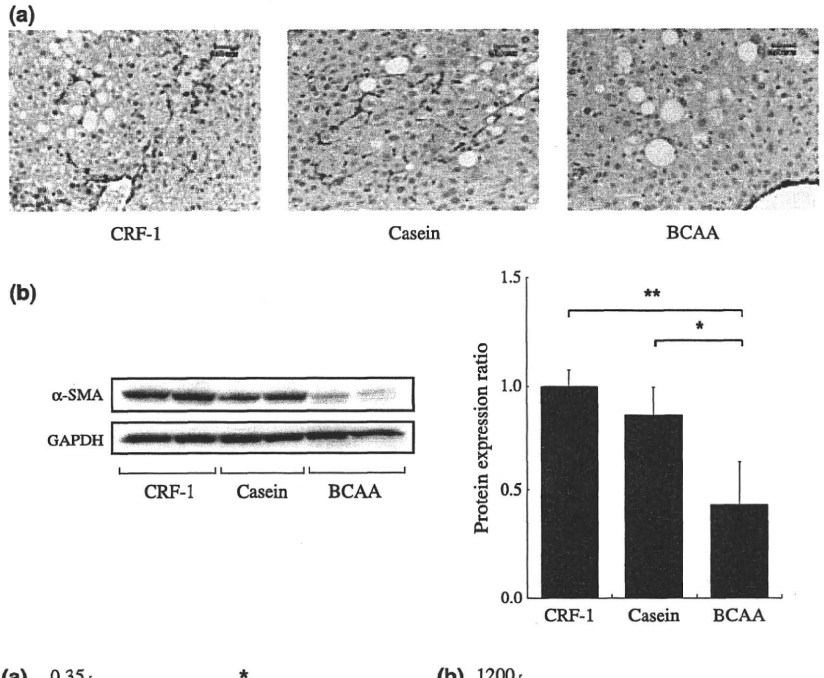

Fig. 6. Effect of branched-chain amino acid (BCAA) supplementation on the expression of α-smooth muscle actin (α-SMA) in diethylnitrosamine-treated C57BL/KsJ-*db/db* mice. (a) Immunohistochemical expression of α-SMA in the liver of basal diet (CRF-1)-fed, casein-supplemented, or BCAA-supplemented treated mice. (b) Total protein was extracted from the liver of experimental mice and the expression of α-SMA protein was examined by Western blot analysis. An antibody to GAPDH served as a loading control. Repeat Western blots gave similar results. The results obtained were quantitated by densitometry and are shown in the right-hand panels. Values are the means ± SD (n = 5). *P < 0.05; **P < 0.01.

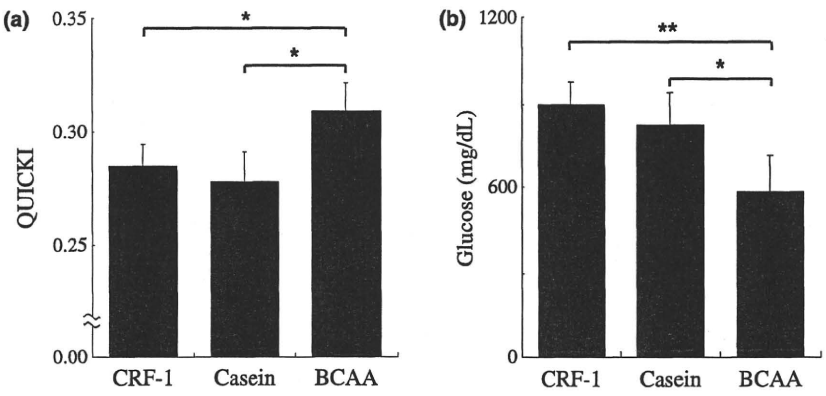

Fig. 7. Effect of branched-chain amino acid (BCAA) supplementation on insulin sensitivity and the serum level of glucose in diethylnitrosamine-treated C57BL/KsJ-*db/db* mice fed basal diet (CRF-1), or supplemented with casein or BCAA. (a) The value of the quantitative insulin sensitivity check index (QUICKI), was calculated to evaluate the insulin sensitivity. (b) The serum concentration of glucose was measured by the hexokinase method. Values are the means ± SD (n = 8). *P < 0.01; **P < 0.001.

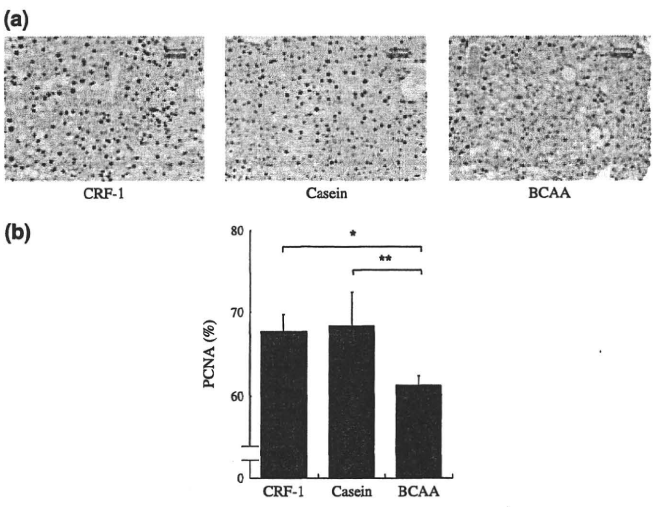

Fig. 8. Effect of branched-chain amino acid (BCAA) supplementation on hepatic cell proliferation in diethylnitrosamine-treated C57BL/KsJ-*db/db* mice. (a) Immunohistochemical expression of proliferating cell nuclear antigen (PCNA) in the liver of basal diet (CRF-1)-fed, or casein- or BCAA-supplemented treated mice. (b) PCNA-labeling index in non-lesional hepatocytes was determined by counting the PCNA-positive nuclei in the hepatocytes. *P < 0.05; **P < 0.01.

severe but did not reach liver cirrhosis at the end point of this experiment (Fig. 5a). This might be explained by a functional defect in the long-form leptin receptor because leptin exerts a pro-fibrogenic activity in the injured liver.[41,42] However, BCAA supplementation significantly decreased the serum levels of leptin (Fig. 3c), inhibited the development of liver fibrosis (Fig. 5), and suppressed the expression of α-SMA (Fig. 6), thus indicating the inhibition of HSC activation. These findings seem to be significant because activated HSCs are a major cellular source of collagen in the injured liver and thus may be a critical target for inhibiting the development of liver fibrosis.[43] Therefore, BCAA supplementation prevents the development of hepatic fibrosis, at least in part, by inhibiting the HSC activation (Fig. 6). In addition, a previous study also indicated that supplementation with BCAA effectively suppressed the hyperleptinemia in *db/db* mice with colonic carcinogenesis model.[17] These findings suggest that leptin is also one of the critical targets of BCAA in obese mice. Future studies would be important to evaluate whether BCAA could also prevent the development of liver fibrosis using a more aggressive fibrotic model, such as methionine and choline-deficient diet-fed *db/db* mice, known to be a good model of progressive NASH.[44]

Finally, it should be emphasized again that, in a recent study, BCAA supplementation in the basal diet was shown to improve insulin resistance, thereby preventing the development of colonic

premalignancies in an obesity-related colon cancer model.[17] Both obesity and insulin resistance are strongly associated with the development of not only HCC, but also colorectal cancer.[45] These previous reports, therefore, further strengthen our conclusion that the prevention of HCC by targeting the dysregulation of energy homeostasis, particularly an increased insulin resistance, might be a promising strategy for obese people who are at increased risk for developing HCC. BCAA appears to be a potentially effective and critical candidate for this purpose because it can improve insulin resistance (Fig. 7a), hepatic steatosis (Fig. 4), and fibrosis (Fig. 5) in obese and diabetic *db/db* mice.

In conclusion, BCAA might therefore represent a new effective strategy for chemoprevention against HCC, especially in obese people. Among the beneficial effects of BCAA shown in this study, the improvement of insulin resistance might play a crucial role to prevent the development of obesity-related liver tumorigenesis because the state of insulin resistance is closely associated with the activation of the IGF/IGF-1R axis, the development of hepatic steatosis and fibrosis.[5–7] In addition, a recent study revealed that BCAA supplementation also suppressed hepatic neovascularization in insulin-resistance-based hepatocarcinogenesis in obese rats.[46]

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Abbreviations

α-SMA	α-smooth muscle actin
ALT	alanine aminotransferase
AOM	azoxymethane
BCAA	branched-chain amino acids
DEN	diethylnitrosamine
FCA	foci of cellular alteration
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HSC	hepatic stellate cell
IGF	insulin-like growth factor
IGF-1R	insulin-like growth factor-1 receptor
NASH	non-alcoholic steatohepatitis
PCNA	proliferating cell nuclear antigen
QUICKI	quantitative insulin sensitivity check index

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Supporting Information

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

Table S1. Incidence and multiplicity of hepatic neoplasms and FCA and serum levels of ALT in db/db, +/+ and B6 mice.

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Review Article

Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis B virus infection for the fiscal year 2008 in Japan

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In the 2008 guidelines for the treatment of patients with cirrhosis, who are infected with hepatitis B virus (HBV), the main goal is to normalize levels of alanine and aspartate aminotransferases by eliminating HBV or reducing viral loads. In patients with compensated cirrhosis, the clearance of HBV from serum is aimed for by entecavir, as the main resort, for histological improvement toward the prevention of hepatocellular carcinoma (HCC). In patients with decompensated cirrhosis, by contrast, meticulous therapeutic strategies are adopted for the reversal to compensation, toward the eventual goal of decreasing the risk of HCC. For maintaining liver function and preventing HCC, branched chain amino acids and nutrient supplements are applied, in addition to conventional liver supportive therapies. For patients with chronic hepatitis B, separate guidelines are applied to those younger than 35 years and those aged 35 years or older. Even for patients with chronic hepatitis who are negative for hepatitis e antigen (HBeAg), but who harbor HBV DNA in titers of 7 log copies/mL or more, a "drug-free state" is aimed for by sequential treatment with interferon (IFN) plus entecavir as the first line. For patients with chronic hepatitis B aged 35 years or older, who are HBeAg-negative and carry HBV DNA in titers of less than 7 log copies/mL, long-term IFN for 24–48 weeks is adopted anew. To HBeAg-negative patients who have either or both platelet counts of less than $150 \times 10^3/mm^3$ and less than 7 log copies of HBV DNA, also, long-term IFN for 24–48 weeks is indicated.

Key words: chronic hepatitis, cirrhosis, hepatitis B virus, hepatocellular carcinoma, interferon, liver supportive therapies, nucleos(t)ide analogs

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INTRODUCTION

SINCE THE FISCAL year 2002, guidelines for the treatment of patients with viral hepatitis have been compiled annually by the Study Group for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis, under the auspice of the Ministry of Health, Labor and Welfare of Japan, supported by enduring efforts of many specialists recruited from all over the nation. Guidelines have been improved every year with many supplementary issues, which had surfaced as our understanding of many facets of viral hepatitis deepened and treatment options widened increasingly with time. For the fiscal year 2008, guidelines have been worked out for a comprehensive standardization of the treatment of chronic hepatitis and cirrhosis due to hepatitis B virus (HBV) and hepatitis C virus (HCV) infections in Japan. These guidelines have been observed by more than 70% of practicing hepatologists treating patients with viral liver disease in Japan. It is hoped that these guidelines will continue being widely accepted and implemented to help as many patients as possible who are suffering from sequelae of persistent hepatitis virus infections.

Here, we relate excerpts of the 2008 guidelines for the treatment of patients with liver disease due to HBV, covering a wide range from those with chronic hepatitis to those with decompensated cirrhosis. The 2008 guidelines for the treatment of liver disease due to HCV are reported in an accompanying paper.

GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS B

PATIENTS WITH CHRONIC hepatitis B can stabilize the activity of liver disease in their natural course, after they have seroconverted from hepatitis B e antigen (HBeAg) to the corresponding antibody (anti-HBe), accompanied by decrease in HBV DNA titers. For that reason, treatment guidelines were constructed separately for the patients younger than 35 years and those aged 35 years or older.

GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS B YOUNGER THAN 35 YEARS

PATIENTS WITH CHRONIC hepatitis B younger than 35 years are treated in accordance with the guidelines summarized in Table 1. Criteria for the treatment eligibility are: (i) serum levels of alanine aminotransferase (ALT) of 31 IU/L or more; and (ii) HBV DNA titers of 5 log copies of more in HBeAg-positive patients and 4 log copies or more in HBeAg-negative patients. In the 2008 guidelines, the indication of treatment is extended to the patients with cirrhosis due to HBV who carry HBV DNA in titers of 3 log copies/mL or more.

In Japan, most HBeAg-positive patients with 7 log copies or more of HBV DNA have been infected with HBV of genotype C by perinatal infection at birth;

Table 1 Guidelines for the treatment of patients with chronic hepatitis B younger than 35 years

Eligibility criteria	ALT	≥31 IU/L
	HBV DNA	HBeAg-positive patients: ≥5 log copies/mL HBeAg-negative patients: ≥4 log copies/mL Patients with cirrhosis: ≥3 log copies/mL
HBV DNA	≥7 log copies/mL	<7 log copies/mL
HBeAg-positive	(1) Long-term IFN for 24–48 weeks (2) Entecavir	(1) Long-term IFN for 24–48 weeks (2) Entecavir
HBeAg-negative	(1) Sequential treatment† (entecavir plus IFN) (2) Entecavir	(1) Regular follow up (2) Long-term IFN for 24 weeks
	Start with entecavir in HBeAg-negative patients who have platelet counts $<15 \times 10^3/mm^3$ and in those with advanced liver disease of stage F2 or higher.	

†Sequential treatment: patients who have lost hepatitis B virus (HBV) DNA after treatment with nucleos(t)ide analogs receive combined interferon (IFN) for 4 weeks, and then IFN monotherapy is continued for 20 weeks, and lifted thereafter. ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen.

Table 2 Guidelines for the treatment of patients with chronic hepatitis B aged 35 years or older

Eligibility criteria	ALT	≥31 IU/L
	HBV DNA	HBeAg-positive patients: ≥5 log copies/mL HBeAg-negative patients: ≥4 log copies/mL Patients with cirrhosis: ≥3 log copies/mL
HBV DNA	≥7 log copies/mL	<7 log copies/mL
HBeAg-positive	(1) Entecavir (2) Sequential treatment† (entecavir plus IFN)	(1) Entecavir (2) Long-term IFN for 24–48 weeks
HBeAg-negative	Entecavir	(1) Entecavir (2) Long-term IFN for 24–48 weeks

†Sequential treatment: patients who have lost hepatitis B virus (HBV) DNA after treatment with nucleot(s)ide analog receive combined interferon (IFN) for 4 weeks, and then IFN monotherapy is continued for 20 weeks, and lifted thereafter. ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen.

accordingly, they would be resistant to interferon (IFN) therapy. Should they receive nucleos(t)ide analogs, however, the duration would become inevitably longer, because they start the treatment when younger than 35 years old. Hence, IFN for 24–48 weeks is the first choice in their treatment. The standard treatment of 3 months is favored, which can be extended to the maximum of 6 months. Non-pegylated (standard) IFN-α is recommended to them, because self-injection at home is approved for preparations of IFN-α; it helps improve their quality of life (QOL). There are many patients who are refractory to IFN and in whom improvement of ALT levels and/or decrease in HBV DNA titers are hardly achievable. Therefore, as another option, monotherapy with entecavir can be applied for the purpose of clearing HBeAg from serum and lowering HBV DNA titers. For HBeAg-positive patients with lower HBV DNA titers (<7 log copies/mL), also, long-term IFN is endorsed as a rule.

There are HBeAg-negative patients in whom ALT levels increase to 31 IU/mL or more repeatedly. In the 2008 guidelines, sequential treatment with IFN and entecavir is introduced as a new arm of therapeutic options for such patients.[1]

For HBeAg-negative patients with less than 7 copies/mL of HBV DNA, in general, regular follow up without therapeutic intervention is deemed to suffice for the majority. For those of them in whom ALT levels flare to 31 IU/mL or more time after time, long-term IFN for 24 weeks is indicated. Because liver disease progresses in many HBeAg-negative patients, for those with platelet counts of less than $150 \times 10^3/mm^3$ or in fibrosis stage F2 or higher, treatment with entecavir is indicated.

GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS B AGED 35 YEARS OR OLDER

TABLE 2 SUMS up treatment modalities for patients with chronic hepatitis B who are aged 35 years or older. HBeAg-positive patients in this age range who carry HBV DNA in titers of 7 log copies/mL or more rarely, if ever, seroconvert to the loss of HBeAg by IFN-based therapies. Hence, entecavir is the first choice in their treatment.[2,3] Because HBV mutants resistant to entecavir can be elicited by it, sequential treatment with IFN plus entecavir is amended in the 2008 guidelines.[1] In view of low viral loads in patients who possess HBV DNA in titers of less than 7 log copies/mL, entecavir is selected as the first choice, followed by long-term IFN as the second choice of treatment in these patients. HBeAg-negative patients who have high viral loads (≥7 log copies/mL), on the other hand, can normalize ALT levels by monotherapy with entecavir. Therefore, entecavir becomes their first choice, and this is the case even in patients with HBV DNA titers less than 7 copies/mL.

GUIDELINES FOR THE TREATMENT WITH NUCLEOS(T)IDE ANALOGS OF PATIENTS WITH CHRONIC HEPATITIS B WHO ARE RECEIVING LAMIVUDINE

TABLE 3 DETAILS guidelines for the treatment with nucleos(t)ide analogs of patients with chronic hepatitis B who are receiving lamivudine. Because a number of drug-resistant HBV mutants emerge increasingly with time in patients on long-term treatment with lamivudine, the fundamental rule is to switch them to ente-

Table 3 Guidelines for the treatment with nucleos(t)ide analogs in patients with chronic hepatitis who are receiving lamivudine

Lamivudine	Less than 3 years		3 years or longer
HBV DNA			
<1.8 log copies/mL persistently	May be switched to entecavir 0.5 mg daily		Continued on lamivudine 100 mg daily
≥1.8 log copies/mL	VBT (−)	May be switched to entecavir 0.5 mg daily	
	VBT (+)	Adefovir 10 mg daily add-on lamivudine	Adefovir 10 mg daily add-on lamivudine

HBV, hepatitis B virus; VBT, virological breakthrough.

cavir. For this reason, patients are stratified by the duration of lamivudine treatment, less than 3 years and 3 years or more, as well as HBV DNA titers persistently below 1.8 log copies/mL and 1.8 log copies/mL or more, and separate treatment strategies have been worked out for the patients in each category. Because by far the majority of patients with a duration of lamivudine treatment of less than 3 years and HBV DNA titers of less than 1.8 copies/mL possess drug-resistant mutants in low frequencies, they are recommended to switch to entecavir 0.5 mg daily as soon as possible. Likewise, patients who have received lamivudine for 3 years or longer, but in whom drug-resistant mutants have never developed, are recommended to switch to entecavir 0.5 mg daily. By contrast, for patients in whom drug-resistant mutants have emerged already and who have undergone virological breakthroughs,[4] adefovir 10 mg daily add-on lamivudine is started for the purpose of stabilizing liver function.[5] In regard of the patients who have received lamivudine for 3 years or longer, those without drug-resistant mutants can stay on lamivudine 100 mg daily.

SUPPLEMENTS TO GUIDELINES FOR THE TREATMENT OF CHRONIC HEPATITIS B (PART I)

FOR THE FISCAL year 2008, the following three items have been added to previous guidelines for the treatment of chronic hepatitis B (Table 4).

1 In the treatment of patients with chronic hepatitis B, IFN is the first resort for those younger than 35 years, toward the eventual goal of gaining a "drug-free state". For the patients aged 35 years or older, persistently negative HBV DNA is the aim of nucleos(t)ide analogs, with the first choice being entecavir in their primary treatment. On the other hand, for patients with HBV mutants resistant to lamivudine and/or entecavir, combined treatment with adefovir and lamivudine is the principal rule (Table 3).[6–8]
2 Therapeutic responses to antiviral treatment are much different in patients with chronic hepatitis B who are infected with HBV of distinct genotypes. It is recommended therefore to determine HBV genotypes before making a decision on the treatment choice. In particular, the patients infected with HBV of genotype A or B respond to IFN in high rates, even if they are aged 35 years or older. For these reasons, IFN becomes the first choice in their antiviral treatment.
3 The duration of IFN treatment is 24 weeks basically. In the patients in whom the efficacy of IFN has been achieved with decrease in HBV DNA titers and normalization of ALT, the treatment duration is better extended to 48 weeks.

Table 4 Supplements to guidelines for the treatment of patients with chronic hepatitis B (part I)

1 Treatment of patients with chronic hepatitis B aims at a "drug-free state" by IFN-based therapies in those younger than 35 years, and at persistently negative HBV DNA in those aged 35 years or older, with entecavir as the first choice in the primary therapy. Lamivudine plus adefovir forms the basis for the treatment of HBV mutants resistant to lamivudine or entecavir.
2 In view of antiviral response much different in patients infected with HBV of distinct genotypes, it is desired to make treatment choices based on genotypes. In particular, because genotypes A and B respond to IFN with high efficacy, even in patients aged 35 years or older, IFN is recommended as the first treatment choice in these patients.
3 The duration of IFN is for 24 weeks basically, but extension to 48 weeks is recommended in patients who respond to IFN with decrease in HBV DNA titers and normalization of ALT levels.

ALT, alanine aminotransferase; HBV, hepatitis B virus; IFN, interferon.