transitions were as follows: rat hepcidin, *m/z* 905.060 → 1118.300; human hepcidin, *m/z* 558.800 → 693.700. Mobile phase A was 0.1 % aqueous formic acid, and mobile phase B was 0.1 % formic acid in acetonitrile.

Statistical analysis

In in vivo study, statistical significances between iron-treated groups and the vehicle group were analyzed by the Tukey test. In in vitro studies, statistical significances were analyzed by Student's *t* test. A P value < 0.05 was used to estimate statistical significance. Data were represented as mean and SD.

Results

SFO administration increased serum hepcidin concentrations in rat

Wistar rats received 2 or 5 mg/kg of SFO as well as vehicle solution. Changes in serum hepcidin concentrations after SFO administration are shown in Fig. 1. Serum hepcidin concentrations in SFO-treated rats were gradually elevated 12 h after SFO injection with subsequent decrease. This effect was dose-dependent and serum hepcidin concentrations in the vehicle group were not increased.

SFO-administrated rat sera increased hepcidin production from HepG2 cells

>We screened hepatocyte-derived cell lines for hepcidin production with various stimuli as previously reported.

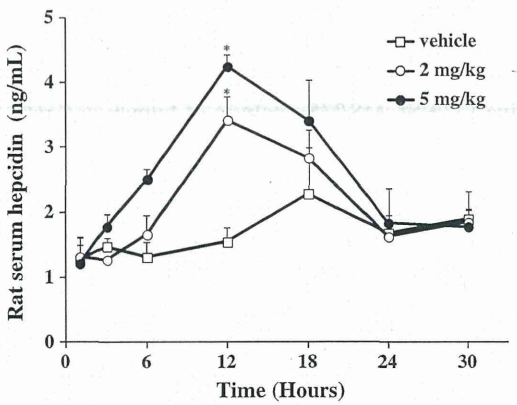

Fig. 1 SFO administration increased serum hepcidin concentrations in rats. Wistar rats received 2 mg/kg (*open circles*) or 5 mg/kg (*filled circles*) of SFO as well as vehicle solution (*open squares*). Serum was analyzed for hepcidin concentration by the LC/ESI–MS/MS method. Results are expressed as mean and SD. Three rats from each group were used. $*P < 0.05$ was found for SFO-treated groups compared with the vehicle group by the Tukey test

None of the tested cell lines produced hepcidin in response to holo-Tf stimulation, while some cell lines, including HepG2, secreted hepcidin after IL-6 stimulation [28]. Among these cell lines, we selected the HepG2 cell for subsequent in vitro experiments because of its higher hepcidin production. Hepcidin production from HepG2 cells was not increased by holo-Tf stimulation, while IL-6 significantly augmented hepcidin production from HepG2 cells as previously reported (Fig. 2a).

To confirm the involvement of humoral factors in hepcidin production induced after SFO administration, we examined the induction of hepcidin by serum from SFO-treated rats or vehicle-treated in HepG2 cells. HepG2 cells were cultured with media containing 10 % (v/v) rat serum for 48 h, and then media were collected and analyzed. As shown in Fig. 2b, SFO-loaded rat serum obviously induced hepcidin production from HepG2, while vehicle-treated rat serum did not induce hepcidin production. Hepcidin production from HepG2 cells stimulated with rat serum correlated with rat serum hepcidin concentrations (Fig. 2c).

Hepcidin concentrations were not correlated with iron indices and cytokines in SFO-administrated rat sera

Serum levels of iron indices and cytokines were examined in SFO-treated rats that have been reported to increase hepcidin production. Serum iron levels were higher in SFO-treated groups than in vehicle-treated group 1 h after SFO injection. Serum iron levels then gradually decreased, and returned to normal levels in both groups (Fig. 3a). NTBI levels were not significantly changed after SFO or vehicle injection (Fig. 3b). Serum IL-6 and IL-1β concentrations, determined by enzyme-linked immunoabsorbent assay (ELISA), were not detected in either serum (data not shown).

Holo-transferrin stimulation increased hepcidin production in co-culture of HepG2 and rat spleen cells

We then hypothesized that the spleen is the putative extra-hepatic iron sensing organ and induces hepcidin production from liver in response to iron, because it is one of the major iron storage sites in the body. HepG2 cells were co-cultured with normal rat spleen cells with or without holo-Tf stimulation, and after 48 h incubation, cultured media were collected and human hepcidin concentrations in the media were analyzed. Although hepcidin production was not up-regulated when HepG2 cells alone were cultured with holo-Tf, holo-Tf stimulation significantly augmented hepcidin production from HepG2 cells co-cultured with rat spleen cells (Fig. 4).

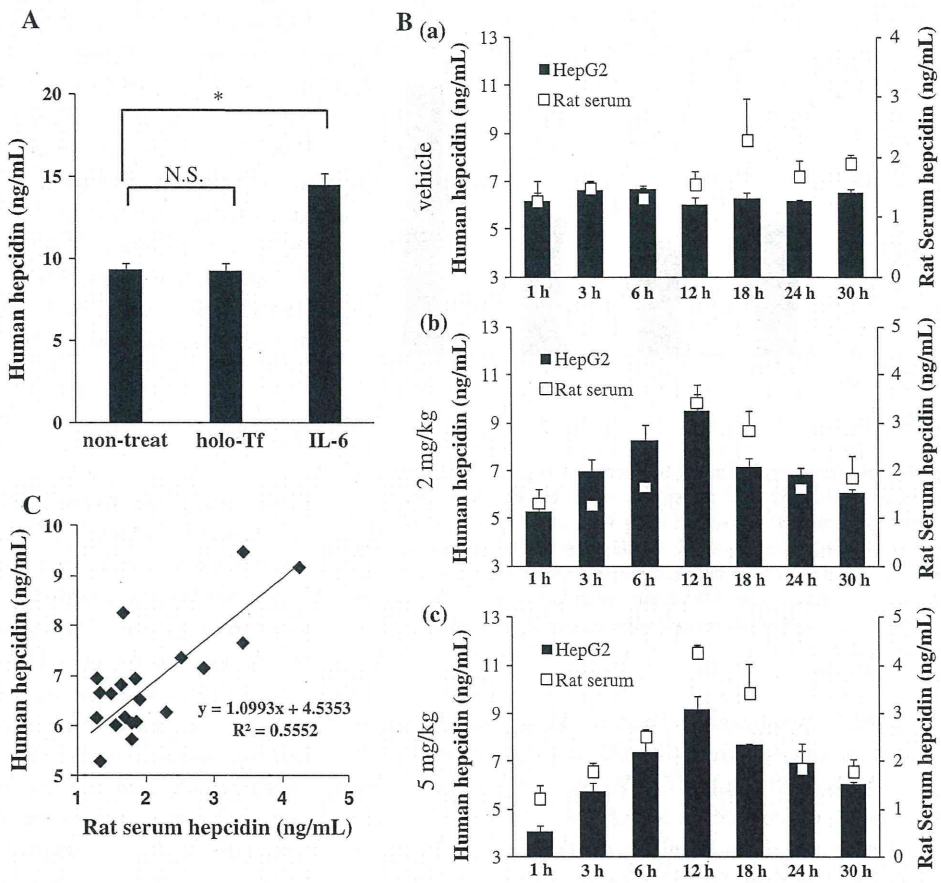

Fig. 2 SFO-administrated rat sera increased hepcidin production from HepG2 cells. **a** HepG2 cells were stimulated with 2.4 mg/mL holo-Tf or 20 ng/mL IL-6 for 48 h, and human hepcidin concentrations in cultured media were analyzed. **b** HepG2 cells were stimulated with 10 % (v/v) rat serum for 48 h, and human hepcidin concentrations in cultured media were analyzed. Results of vehicle-treated rats (*a*), 2 mg/kg SFO-treated rats (*b*), or 5 mg/kg SFO-treated rats (*c*) are shown. **c** The figure shows the correlation between rat serum hepcidin concentrations and hepcidin production from HepG2 cells incubated with rat serum. Results are expressed as mean and SD. Each treatment was performed in triplicate. Statistical significances were analyzed by Student's *t* test with a P value < 0.05

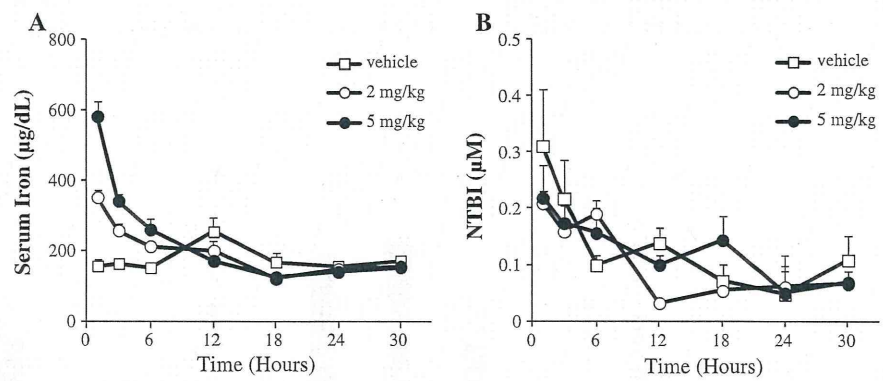

Fig. 3 Iron indices transitions in SFO-administrated rat sera were independent from hepcidin concentrations. Wistar rats received 2 mg/kg (*open circles*) or 5 mg/kg (*filled circles*) of SFO as well as vehicle solution (*open squares*). Serum iron levels (**a**) and serum NTBI levels (**b**) in each-treated rat sera were analyzed. Results are expressed as mean and SD. Three rats from each group were used

Holo-transferrin stimulation increased hepcidin production in co-culture of HepG2 and THP-1 through humoral factors

To determine which cells in spleen affect hepcidin production in response to iron, we next tested whether reticuloendothelial monocytes are iron sensing cells, because spleen produces and stores monocytes [29] and monocytes are known to be involved in modulation of iron metabolism [30, 31]. We selected the human monocytic cell line, THP-1, for subsequent experiments. We observed the expression of CD71 which is needed for cellular import of iron by flow cytometry (FCM). An increase of iron uptake in THP-1 cells after addition of holo-Tf was detected by inductively coupled plasma atomic emission spectroscopy (data not shown). HepG2 cells were co-cultured with THP-1 cells with or without holo-Tf stimulation. After 48 h incubation, cultured media were collected and human hepcidin concentrations in the media were analyzed. As shown in Fig. 5a, hepcidin production from HepG2 cells was up-regulated by coexistence of THP-1 cells, while holo-Tf stimulation further increased hepcidin production from

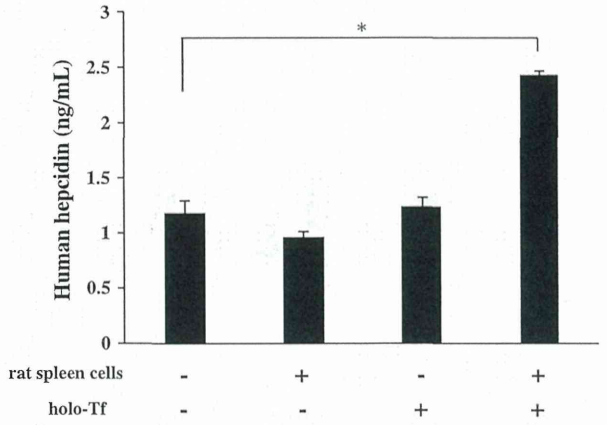

Fig. 4 Holo-transferrin stimulation increased hepcidin production in co-culture of HepG2 and rat spleen cells. HepG2 cells were co-cultured with normal rat spleen cells with or without holo-Tf, and after 48 h incubation, cultured media were collected and human hepcidin concentrations in cultured media were analyzed. Results are expressed as mean and SD. Each treatment was performed in triplicate. Statistical significances were analyzed by Student's *t* test with a *P* value < 0.05

HepG2 cells significantly. Tocilizumab could not inhibit hepcidin production from HepG2 cells co-cultured with THP-1 cells with holo-Tf. We next investigated the requirement of cell–cell contact for cross-talk between HepG2 cells and THP-1 cells in mediating hepcidin regulation. As shown in Fig. 5b, we found up-regulation of hepcidin production from HepG2 cells with holo-Tf stimulation in noncontact system of HepG2 cells and THP-1 cells.

Holo-transferrin stimulation increased hepcidin production in co-culture of HepG2 and human peripheral blood monocytes

HepG2 cells were co-cultured with human peripheral blood monocytes with or without holo-Tf stimulation. After 48 h incubation, cultured media were collected and human hepcidin concentrations in the media were analyzed. Holo-Tf stimulation augmented hepcidin production from HepG2 cells co-cultured with human peripheral blood monocytes significantly (Fig. 6).

Discussion

In this study, we investigated the mechanism of hepcidin production in response to iron to clarify the existence of putative extra-hepatic iron sensors.

Several groups reported that iron loading leads to elevated transcription of the *HAMP* gene and increased serum hepcidin levels in vivo [18, 19]. We detected elevated hepcidin levels in rat serum after SFO administration (Fig. 1). We have established a method for measuring hepcidin levels in media of cultured cell lines. In screened hepatocyte-derived cell lines, none of the tested cells up-regulated hepcidin production following holo-Tf addition, although IL-6 up-regulated hepcidin production and N-terminally truncated hepcidin isoforms were increased by holo-Tf in some cell lines [28]. These findings lead us to hypothesize that these cell lines lack iron sensory system so that the iron loading signal is mediated by humoral factors

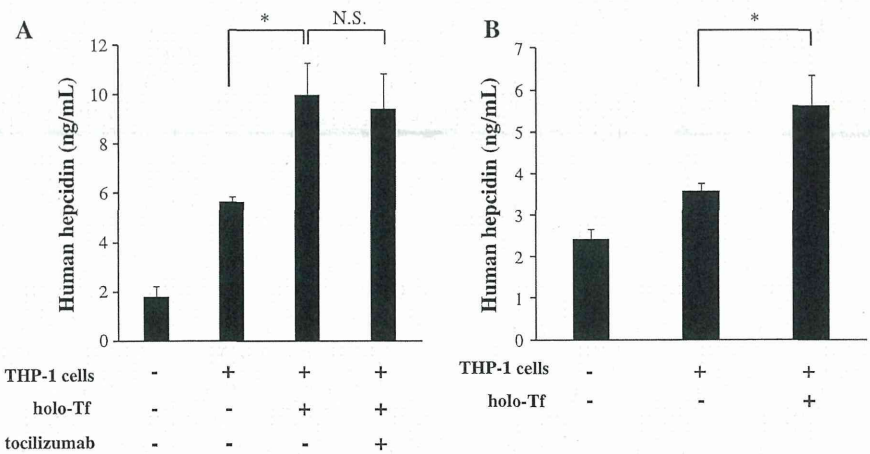

Fig. 5 Holo-transferrin stimulation increased hepcidin production in co-culture of HepG2 and THP-1 through humoral factors. **a** HepG2 cells were co-cultured with THP-1 cells with holo-Tf alone or in combination with tocilizumab. After 48 h incubation, cultured media were collected and human hepcidin concentrations in cultured media were analyzed. **b** HepG2 cells were co-cultured with THP-1 cells with or without holo-Tf using cell culture inserts to inhibit cell–cell contact. After 48 h incubation, cultured media were collected and human hepcidin concentrations in cultured media were analyzed. Results are expressed as mean and SD. Each treatment was performed in triplicate. Statistical significances were analyzed by Student's *t* test with a *P* value < 0.05

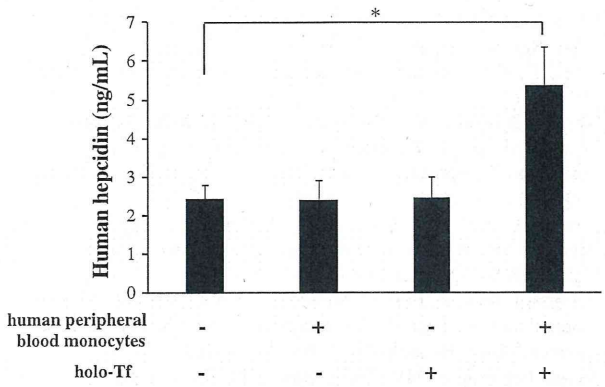

Fig. 6 Holo-transferrin stimulation increased hepcidin production in co-culture of HepG2 and human peripheral blood monocytes. HepG2 cells were co-cultured with human peripheral blood monocytes with or without holo-Tf, and after 48 h incubation, cultured media were collected and human hepcidin concentrations in cultured media were analyzed. Results are expressed as mean and SD. Each treatment was performed in triplicate. Statistical significances were analyzed by Student's t test with a P value < 0.05

such as cytokines like BMP participating in iron homeostasis.

To confirm the involvement of humoral factors induced by SFO administration in hepcidin production, we examined levels of hepcidin in HepG2 cells cultured in serum from SFO-treated rats. As shown in Fig. 2b, SFO-loaded rat serum significantly induced hepcidin production from HepG2 cells. The LC/ESI–MS/MS method for hepcidin detection, which we have established, allows discriminating human hepcidin from rat hepcidin from the difference between the molecular masses of precursor and product ions. Rat hepcidin in rat serum did not interfere with quantification of human hepcidin (data not shown). Hepcidin production from HepG2 cells stimulated with rat serum was correlated with rat serum hepcidin concentrations (Fig. 2c), but not correlated with iron indices and cytokines such as IL-6 and IL-1β that have been reported to induce hepcidin production (Fig. 3). These results suggest that humoral factors, whose relation with hepcidin regulation have previously been unsuspected, mediate iron loading signals for up-regulation of hepcidin; iron sensors may therefore be located on extra-hepatic cells.

It has been reported that an indirect mechanism may function for the up-regulation of hepatic hepcidin expression by iron [32]. THP-1 cells were differentiated into macrophages with phorbol 12-myristate 13-acetate (PMA), and conditioned medium was collected after holo-Tf stimulation for 24 h. Hepcidin mRNA expression levels in HepG2 cells with activated THP-1 conditioned medium were up-regulated suggesting that iron stimulation induces hepcidin mRNA synthesis from hepatocyte via cross-talk with macrophages. Another report suggests that IL-1β is the predominant macrophage factor involved in inducing hepatic *HAMP* expression. Cross-talk between macrophages and hepatocytes may induce hepcidin expression, with these two cell types taking an active role in regulating hepcidin during inflammation [33]. Macrophages are produced by differentiation of monocytes in damaged tissues and have a low chemotactic activity. We therefore hypothesize that extra-hepatic iron sensors maintain the iron homeostasis. Reticuloendothelial monocytes circulate in the bloodstream and are known to be involved in modulation of iron metabolism.

The spleen is a site for storage and rapid development of monocytes [29]. HepG2 cells were co-cultured with normal rat spleen cells with or without holo-Tf stimulation. Holo-Tf increased hepcidin production in a co-culture system (Fig. 4). A human monocytic cell line, THP-1, was chosen as a model of reticuloendothelial monocytes model expressing transferrin receptor CD71 for subsequent experiments. These cells took up iron after addition of holo-Tf (data not shown). THP-1 did not produce hepcidin in transcriptional and protein levels (data not shown). HepG2 cells were co-cultured with non-activated THP-1 cells with or without holo-Tf. Holo-Tf increased hepcidin production in the co-culture system both with and without cell–cell contact (Fig. 5). These experiments showed that direct cell–cell contact between HepG2 cells and THP-1 cells was not required for hepcidin production in response to iron. Humoral factors increasing hepatic hepcidin production in response to iron might be secreted into blood. In this co-cultured system, tocilizumab could not inhibit hepcidin production from HepG2 cells co-cultured with THP-1 cells with holo-Tf, while induction of hepcidin production in response to iron was independent of IL-6 signaling. Finally, HepG2 cells co-cultured with human peripheral blood monocytes with or without holo-Tf showed that holo-Tf increased hepcidin production in the co-culture system (Fig. 6).

A molecular mechanism of hepcidin regulation by iron was recently reported [34]. Extracellular iron in the form of holo-Tf binds with transferrin receptor (TfR)1 and TfR2, and TfR2 is known to be one of the key iron sensors [35]. TfR2 is highly expressed in the liver, and faintly expressed in spleen and bone marrow [36]. TfR2 associates with HFE, which is essential for hepcidin expression, and BMP signaling via Sma- and Mad-related proteins (SMADs) for hepcidin induction is modulated by these components [37–39]. We observed the expression of TfR2 as well as HFE in HepG2 cells. Hemojuvelin (HJV) expression was also detected by quantitative PCR (data not shown). Hepcidin production from HepG2 cells was induced in response to BMP2 [28], indicating that HepG2 cells must express all components for iron sensing except for humoral factors secreted in response to iron. BMP6 is also thought to be a key endogenous regulator of hepcidin expression and iron metabolism [22, 23].

BMP6 interacts with HJV and BMP receptors, and induces hepcidin through translocation of SMADs to the nucleus. BMP6 is believed to be expressed mainly in the liver for sensing intracellular iron [24, 40]. We checked BMP6 protein levels in media from HepG2 cells co-cultured with THP-1 cells with or without holo-Tf, but BMP6 levels needed to increase hepcidin production from HepG2 cells were not detected (data not shown). Thus, other factors than BMP6 and IL-6 may be secreted from monocytes in response to iron to induce hepcidin production, and detailed mechanisms should be clarified in the future.

Various assays have been developed for quantification of hepcidin on mass spectrometry technique including surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF–MS) and LC/ESI–MS/MS [27, 41, 42]. Recently, immunochemical assays such as competitive radio-immunoassays (RIA) and ELISA to measure hepcidin have also been developed [13, 43, 44]. Hepcidin has the potential to be a novel clinical biomarker in iron metabolism disorders, because the involvement of hepcidin detected in blood and urine in the dysregulation of iron homeostasis in various disease, such as CKD, hereditary hemochromatosis and hepatitis C virus (HCV) infection has been reported [45–47]. On the other hand, hepcidin may reflect not only iron metabolism disorders but also other disease states, because hepcidin expression is controlled by several stimuli such as inflammation. Monocyte-derived humoral factors we suggested are thought to be iron-responsive, and then these factors are believed to allow for more elaborate diagnosis of iron metabolism disorders and develop into therapeutic target for them.

In conclusion, our results indicate the existence of humoral factors secreted in response to iron loading which could induce hepcidin production was clarified. Our data also suggested that they were secreted by extra-hepatic cells, such as reticuloendothelial monocytes to maintain physiological iron homeostasis. The nature of the factors awaits future identification.

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Conflict of interest The authors declare that they have no conflict of interest.

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Branched-Chain Amino Acids Prevent Hepatocarcinogenesis and Prolong Survival of Patients With Cirrhosis

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BACKGROUND & AIMS: **Although a low plasma level of branched-chain amino acids (BCAAs) is a marker of cirrhosis, it is not clear whether BCAA supplements affect disease progression. We performed a multicenter study to evaluate the effects of BCAA supplementation on hepatocarcinogenesis and survival in patients with cirrhosis.**

METHODS: **We enrolled 299 patients from 14 medical institutions in Japan in a prospective, multicenter study in 2009; 267 patients were followed through 2011. Patients were given BCAA supplements (5.5–12.0 g/day) for more than 2 years (n = 85) or no BCAAs (controls, n = 182). The primary end points were onset of hepatocellular carcinoma (HCC) and death. Factors associated with these events were analyzed by competing risk analysis.**

RESULTS: **During the study period, 41 of 182 controls and 11 of 85 patients given BCAAs developed HCC. On the basis of the Cox and the Fine and Gray models of regression analyses, level of α-fetoprotein, ratio of BCAA:tyrosine, and BCAA supplementation were associated with development of HCC (relative risk for BCAAs, 0.45; 95% confidence interval, 0.24–0.88; $P = .019$). Sixteen controls and 2 patients given BCAAs died. Factors significantly associated with death were Child–Pugh score, blood level of urea nitrogen, platelet count, male sex, and BCAA supplementation (relative risk of death for BCAAs, 0.009; 95% confidence interval, 0.0002–0.365; $P = .015$) in both regression models.**

Abbreviations used in this paper: AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCAA, branched-chain amino acid; BMI, body mass index; BUN, blood urea nitrogen; CT, computed tomography; HbA1c, glycosylated hemoglobin; HCC, hepatocellular carcinoma; HOMA-IR, homeostasis model assessment of insulin resistance; MRI, magnetic resonance imaging; TIBC, total iron binding capacity.

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CONCLUSIONS: **On the basis of a prospective study, amino acid imbalance is a significant risk factor for the onset of HCC in patients with cirrhosis. BCAA supplementation reduces the risk for HCC and prolongs survival of patients with cirrhosis.**

Keywords: Liver Cancer; Hepatoma; Nutrition; Treatment Outcome.

The liver is a central organ in the metabolism of many nutrients. Thus, cirrhosis of the liver frequently results in metabolic disarray.[1,2] Decreased serum levels of branched-chain amino acids (BCAAs) are a hallmark of cirrhosis[1,2] that are contributed to by several factors, including reduced nutritional intake, hypermetabolism, and ammonia detoxification in skeletal muscle.[3] Low serum levels of BCAAs are also important in the pathogenesis of hepatic encephalopathy and hypoalbuminemia and are associated with overall mortality.[1–3]

BCAAs are a source of glutamate, which detoxifies ammonia via glutamine synthesis in skeletal muscle.[4] BCAAs have recently been considered as pharmacologic nutrients in cirrhotic patients.[1] In vitro studies have demonstrated that BCAAs prevent the proliferation of hepatocellular carcinoma (HCC) cells by inducing apoptosis.[5] In addition, BCAA supplementation was shown to stimulate antioxidant DNA repair in a rat model of liver injury[6] and to prevent hepatocarcinogenesis in an animal model.[7] In HCC patients, BCAA supplementation reduces early recurrence of HCC after hepatic resection or radiofrequency thermal ablation.[8,9] To investigate the effects of BCAA supplementation on the development of cirrhosis-related complications, multicenter randomized controlled trials were conducted in Italy and Japan at the end of the 1990s.[10,11] Although these studies showed that BCAA supplementation prevented hospital admissions related to cirrhosis complications and improved the quality of life of cirrhotic patients,[10,11] the effects of BCAA supplementation on hepatocarcinogenesis remain unclear.

BCAAs are also known to enhance hepatic regeneration and immunity.[1,2] BCAAs stimulate the production of hepatocyte growth factor in hepatic stellate cells[12] and increase hepatic parenchymal cell mass.[13] In addition, BCAA supplementation increases lymphocyte counts and improves the phagocytic function of neutrophils in cirrhotic patients.[14] BCAAs also reverse functional impairment and stimulate the maturation of myeloid dendritic cells, leading to the production of interleukin-12, a potent activator of natural killer cells.[15] Recently, bacterial infection has become one of the major causes of death in cirrhotic patients.[16] Taken together, these findings suggest that BCAA supplementation may prevent hepatic failure and bacterial infection, leading to prolonged survival in cirrhotic patients. Hitherto, a survival benefit of BCAA supplementation has not been demonstrated.[17]

In Japan, BCAA supplementation is an approved medication for decompensated liver cirrhosis, and thus, a randomized control trial that uses BCAA supplementation cannot ethically be performed. Moreover, the onset of HCC and death are considered as competing risks. Therefore, the aim of this study was to evaluate the effects of BCAA supplementation on the onset of HCC and survival in cirrhotic patients by competing risk analysis.

Methods

Study Design and Ethics

This study was designed in 2009 by the steering committee as a multicenter investigation for evaluating the effects of BCAA supplementation on hepatocarcinogenesis and prognosis in cirrhotic patients. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in the prior approval given by the institutional review board of each institution. None of the subjects were institutionalized.

Subjects and Observation Period

In 2009, 299 cirrhotic patients without HCC were enrolled from 14 medical institutions in Japan. Diagnosis of liver cirrhosis was based on an aspartate aminotransferase (AST)-to-platelet ratio index >1.5,[18] morphologic changes of the liver such as hypertrophy of the left lateral and caudate lobes and atrophy of the right posterior hepatic lobe as evidenced by ultrasonography, computed tomography (CT), and/or magnetic resonance imaging (MRI), or a pseudo-lobule formation finding on histopathologic examination. The etiologies of liver cirrhosis were hepatitis C virus infection (n = 171), hepatitis B virus infection (n = 31), alcohol intake (n = 24), autoimmune hepatitis (n = 22), nonalcoholic fatty liver disease (n = 12), and others (n = 7).

Enrolled patients were followed up until 2011. The median observation period was 728 days (range, 22–1069 days), and the mean observation period was 677.9 ± 220.0 days. During the course of the study, 32 patients could not be followed up because of a change of residence (n = 12), failure to attend appointments (n = 17), or data unavailability (n = 3). The remaining 267 cirrhotic patients were analyzed (follow-up rate, 89.3%) (Supplementary Figure 1). A total of 16 patients had hepatic encephalopathy at study entry and were treated with BCAA supplementation, and 4 patients developed hepatic encephalopathy during the study period.

Classification

BCAA supplementation (BCAA granules and BCAA-enriched nutrients) is an approved medication for decompensated liver cirrhosis in Japan. Thus, according to the indication criteria, BCAAs were administered to cirrhotic patients with hepatic encephalopathy or hypoalbuminemia. Patients treated with BCAA supplementation (5.5–12.0 g/day) for >2 years were classified as the BCAA group (n = 85), whereas those without hepatic encephalopathy or hypoalbuminemia (n = 152) or in whom administration was difficult (n = 30) were classified as the non-BCAA group (n = 182). The reasons for BCAA administration difficulty were noncompliance because of the bitterness of the supplement or supplement-related adverse effects such as gastrointestinal discomfort and diarrhea.

Definition of an Event

In this study, an event was defined as the onset of HCC or death from any cause. Subjects were regularly followed up by doctors specializing in liver disease. The follow-up examinations included routine physical examinations, biochemical tests, and HCC screening with 4 monthly tests of serum α-fetoprotein (AFP) levels and diagnostic imaging studies including ultrasonography, CT, or MRI.

HCC was diagnosed by using a combination of the levels of serum tumor markers, such as AFP and des-gamma-carboxy prothrombin, and findings of imaging studies such as ultrasonography, CT, MRI, and angiography. For deceased subjects, the disease directly causing death was defined as the cause of death. Chronic hepatic failure was defined as having jaundice, refractory ascites, and/or hepatic encephalopathy. No patient died of acute liver failure or acute chronic liver failure.

A censoring case was defined as a subject who was followed up until the end of the study period without onset of HCC or death.

Data Collection

Data on the following parameters were collected at study entry: age, sex, daily alcohol intake (none, <60 g, or >60 g), body mass index (BMI), platelet count, serum levels of AST, alanine aminotransferase (ALT), albumin, total bilirubin, total cholesterol, triglycerides, fasting blood glucose, insulin, blood urea nitrogen (BUN), creatinine, sodium, potassium, zinc, iron, total iron binding capacity (TIBC), ferritin, AFP, prothrombin time, proportion of glycosylated hemoglobin (HbA1c), homeostasis model assessment of insulin resistance (HOMA-IR), blood ammonia level, BCAA-to-tyrosine ratio, and Child–Pugh score. Intake of BCAA supplementation was evaluated at every visit.

Statistics

Differences between the BCAA and non-BCAA groups were analyzed by using the Wilcoxon rank sum test. Factors associated with the onset of HCC and death were analyzed by competing risk analysis as previously described.[19]

Bivariate analyses were performed by using the multiple Cox regression model with stepwise selection (enter and exit probabilities are $P = .05$) for the cause-specific hazard of HCC onset and death and the Fine and Gray regression model for the subdistribution hazard of HCC onset and death. Covariates were selected by using a stepwise procedure adapted to multiple imputation methodology.[19] The Fine and Gray model provides complementary competing risk data to the Cox proportional hazards model by considering the subdistribution hazard.[19] P values <.05 were considered significant. All analyses were performed by using the R statistical programming language and computing environment with survival, cmprsk, and MICE packages.[20]

Results

Patient Characteristics

At baseline, no significant differences were noted in age, sex, daily alcohol intake, BMI, platelet count, fasting blood glucose levels, HOMA-IR values, and serum levels of AST, ALT, creatinine, sodium, and AFP between the BCAA and non-BCAA groups (Table 1). In contrast, serum levels of albumin, total cholesterol, ferritin, and the BCAA-to-tyrosine ratio were significantly lower in the BCAA group than in the non-BCAA group (Table 1). In addition, blood ammonia levels and Child–Pugh scores were significantly higher in the BCAA group than in the non-BCAA group (Table 1).

Incidence Rate of Events

During the study, 52 patients developed HCC (41 patients in the non-BCAA group and 11 patients in the BCAA group), and 18 patients died (16 patients in the non-BCAA group and 2 patients in the BCAA group) (Table 2). The incidence rates of HCC onset and death in the overall study sample were 19.5% (52 of 267) and 6.7% (18 of 267), respectively.

The causes of death and the incidence rates for each group are summarized in Supplementary Table 1. Chronic hepatic failure and bacterial infection accounted for 27.8% (5 of 18) and 22.2% (4 of 18) of all deaths, respectively. Of the 16 deaths in the non-BCAA group, 4 (25%) were due to hepatic failure, and 4 (25%) were due to bacterial infection. Of the 2 deaths in the BCAA group, 1 (50%) was due to hepatic failure, and none were due to bacterial infection (Supplementary Table 1).

Table 1. Patient Characteristics

	Non-BCAA group	BCAA group	*P* value
n	182	85	
Age (*y*)	67.0 (35–89)	68.0 (31–86)	.817
Sex (female/male)	101/81	44/41	.569
Daily alcohol intake (none/<60 g/≥60 g) (%)	81.0/11.3/7.7	80.0/8.0/12.0	.450
BMI (kg/m^2)	22.9 (14.1–32.9)	23.9 (16.2–36.7)	.137
Platelet count ($\times 10^3/mm^3$)	92.5 (24.0–430.0)	85.0 (29.0–357.0)	.931
AST (*IU/L*)	41 (17–195)	48 (18–198)	.160
ALT (*IU/L*)	35 (7–173)	32 (9–100)	.762
Albumin (*g/dL*)	3.75 (2.31–4.95)	3.30 (1.99–4.20)	<.001
Prothrombin time (international normalized ratio)	1.08 (0.47–1.85)	1.14 (0.55–1.99)	.109
Total bilirubin (*mg/dL*)	1.00 (0.28–2.90)	1.10 (0.30–2.90)	.365
Total cholesterol (*mg/dL*)	157 (77–280)	142 (81–258)	.012
Triglycerides (*mg/dL*)	82 (30–367)	78 (25–233)	.449
Fasting blood glucose (*mg/dL*)	106 (55–280)	107 (73–256)	.360
HbA1c (%)	5.2 (4.0–9.9)	5.2 (4.6–9.5)	.808
Fasting insulin (*μU/mL*)	12.0 (2.0–167)	13.3 (3.6–106.3)	.072
HOMA-IR	3.3 (0.4–20.1)	4.0 (0.7–36.9)	.812
Ammonia (*μg/dL*)	38 (4–245)	54 (6–326)	<.001
BCAA-to-tyrosine ratio	3.71 (1.36–12.6)	2.87 (1.08–8.26)	<.001
Child–Pugh score	6 (5–10)	7 (5–11)	<.001
BUN (*mg/dL*)	15.0 (3–140)	16.0 (0.6–41)	.886
Creatinine (*mg/dL*)	0.70 (0.37–7.9)	0.72 (0.41–1.73)	.111
Sodium (*mEq/L*)	141 (124–148)	141 (131–146)	.930
Potassium (*mEq/L*)	4.1 (2.5–5.6)	4.2 (3.4–5.1)	.477
Zinc (*μg/dL*)	64 (38–116)	61 (21–116)	.210
Iron (*μg/dL*)	115 (22–332)	103 (17–274)	.107
TIBC (*μg/dL*)	344 (107–563)	328 (174–538)	.608
Ferritin (*ng/mL*)	90.4 (4.3–1624.7)	58.2 (4.0–478.4)	.023
AFP (*ng/mL*)	6.4 (0.6–191.0)	6.4 (1.0–188.6)	.351

NOTE. Data are expressed as number or median (ranges). Differences between the 2 groups were analyzed by using the Wilcoxon rank-sum test. *P* values <.05 were considered significant.

Competing Risk Analysis for the Onset of Hepatocellular Carcinoma and Death

In a multiple Cox regression analysis, serum AFP and TIBC levels were positive risk factors significantly associated with the onset of HCC. An increase in serum BCAA-to-tyrosine ratio was a negative risk factor significantly associated with the onset of HCC. These risk factors were also significantly associated with the onset of HCC in the Fine and Gray analysis (Table 3). Moreover, intake of BCAA supplementation was a negative risk factor significantly associated with the onset of HCC in both the multiple Cox and Fine and Gray analyses ($P = .026$ and $P = .019$, Table 3) after adjusting for other covariates.

Table 2. Cumulative Incidence of Events

	n	All subjects (n = 267)	Non-BCAA group (n = 182)	BCAA group (n = 85)
HCC	52	19.5% (52/267)	22.5% (41/182)	12.9% (11/85)
Death	18	6.7% (18/267)	8.8% (16/182)	2.4% (2/85)

In the multiple Cox regression analysis, the following 6 factors were significantly associated with death: Child–Pugh score, serum BUN level, platelet count, male sex, serum iron level, and HOMA-IR value. However, serum iron level and HOMA-IR value were not significantly associated with death in the Fine and Gray analysis (Table 3). In both the multiple Cox and Fine and Gray analyses, intake of BCAA supplementation was a negative risk factor significantly associated with death ($P = .007$ and $P = .015$, Table 3) after adjusting for other covariates.

Cumulative Incidence of Hepatocellular Carcinoma and Death Between the Branched-Chain Amino Acids and Non–Branched-Chain Amino Acids Groups

Cumulative incidence of the onset of HCC was significantly lower in the BCAA group compared with that in the non-BCAA group (relative risk, 0.45; 95% confidence interval, 0.24–0.88; $P = .019$) (red lines in Figure 1). Cumulative incidence of death was also significantly lower in the BCAA group compared with that in the non-BCAA group (relative risk, 0.009; 95% confidence interval, 0.0002–0.365; $P = .015$) (black lines in Figure 1).