Treatment with L-Valine Ameliorates Liver Fibrosis and Restores Thrombopoiesis in Rats Exposed to Carbon Tetrachloride

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It has been reported that treatment with branched chain amino acids (BCAAs) increases the survival rates in cirrhotic patients. In this study, we investigated the effect of L-valine, one of BCAAs, on liver fibrosis in rat. To induce liver fibrosis, male Wistar rats were injected carbon tetrachloride (CCl4) intraperitoneally (2.0 mL/kg) twice a week for 12 weeks. The rats (seven to fifteen rats for each group) were then administered 1.688 g/kg/day of L-valine intravenously for 7 days or 10% amino acid preparation that provided the same amount of nitrogen. Seven days after the last administration, blood platelet counts and bone marrow megakaryocyte counts were significantly higher in the valine group than in the control group (131.2 ± 38.3 vs. 106.3 ± 14.5 × 10³/µL, p = 0.04; 18.0 ± 2.1 vs. 13.5 ± 2.2 per field, p < 0.01, respectively). Importantly, the mRNA level of thrombopoietin, a key regulator of thrombopoiesis, was significantly higher in the liver of the valine group than the control group. Furthermore, hepatic fibrosis was significantly reduced in the valine group, and the mRNA levels of factors associated with liver fibrosis such as procollagen α1(III), transforming growth factor-β1 and connective tissue growth factor were significantly lower in the liver of the valine group 10 days after the last administration. These results indicate that L-valine treatment ameliorates liver fibrosis and restores thrombopoiesis in rats exposed to CCl4. Therefore, L-valine supplementation may be helpful for patients with liver cirrhosis.

Keywords: valine; thrombopoietin; liver fibrosis; TGF-β; CTGF

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In cirrhotic patients, it is known that serum levels of branched chain amino acids (BCAAs: valine, leucine and isoleucine) and Fischer’s BCAA/AAA (aromatic amino acids: phenylalanine, tyrosine and tryptophan) molar ratio are reduced (Morgan et al. 1978). BCAAs have been administered orally to effectively reverse hypoalbuminemia and hepatic encephalopathy in patients with liver cirrhosis (Eriksson et al. 1982; Marchesini et al. 2003). Furthermore, administration of BCAAs has been reported to prevent or retard the progression of liver failure in cirrhotic patients (Marchesini et al. 2000, 2003; Muto et al. 2005). However, the mechanism that BCAAs generate the observed effects is not clearly understood.

The development of liver fibrosis involves a marked accumulation of extra cellular matrix (ECM) components, activation of cells capable of producing matrix materials, cytokine release, and tissue remodeling. Transforming growth factor-β1 (TGF-β1) is closely associated with liver fibrosis (Czaja et al. 1989) and induces collagen formation by increasing procollagen mRNA levels (Ignotz et al. 1987; Raghow et al. 1987). In chronic liver injury, TGF-β1 secreted by Kupffer cells stimulates the proliferation and induces the activation of hepatic stellate cells. The activated hepatic stellate cells play a crucial role in the development of liver fibrosis through the production of several ECM components (Friedman 2000; Ozaki et al. 2002; Rippe and Brenner 2004). Furthermore, connective tissue growth factor (CTGF), which stimulates fibroblast proliferation and ECM synthesis, is strongly expressed during liver fibrogenesis (Paradis et al. 1999).

Thrombopoietin (ThPO) has been identified as a key regulator of platelet production. ThPO mRNA is expressed mainly in hepatocytes (Nomura et al. 1997; Wolber et al. 1999a). Both human and animal studies have shown that ThPO mRNA levels in liver, kidney, and spleen are not influenced by the concentration of circulating platelets (Sungaran et al. 1997). Thrombocytopenia is frequent in patients with liver cirrhosis, and is thought to be largely due to hypersplenism. However, thrombocytopenia associated with liver cirrhosis may be attributable to reduced hepatic ThPO mRNA levels and a relatively low plasma ThPO concentration (Martin et al. 1997; Peck-Radosavljevic et al. 1997; Qian et al. 1998; Wolber et al. 1999b; Giannini et al. 2003). In another study, hepatocyte growth factor (HGF)
increased platelet production through an enhancement of ThPO mRNA expression in the liver of cirrhotic rats (Yamashita et al. 2000). The expression of ThPO and platelet counts are more increased in transgenic mice liver overexpressing murine HGF compared to the wild type (Kosone et al. 2007). Recently, a phase 2 multicenter, randomized study showed that etrombopag, a small-molecule nonpeptide ThPO receptor agonist, increased platelet counts in patients with hepatitis C virus-associated thrombocytopenia and compensated liver disease (McHutchison et al. 2007). Furthermore, it has been suggested that platelets itself have effects on improvement of liver fibrosis (Watanabe et al. 2009).

To the best of our knowledge, there has been no report about the impact of BCAAs on platelets counts and ThPO production in the cirrhotic liver. Furthermore, the effect of L-valine, one of the BCAAs, on the liver fibrosis is not well understood (Marchesini et al. 2005). In this study, we investigated whether L-valine therapy ameliorates liver fibrosis and restores thrombopoiesis in rats with carbon tetrachloride (CCl₄)-induced cirrhosis.

**Materials and Methods**

**Animal experiments**

Specific pathogen-free male Wistar rats aged 7 weeks (weighing approximately 200 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). All animals received humane care and the experimental protocol was approved by the Committee of Laboratory Animals according to the guideline of Tohoku University. All animals were maintained in a temperature controlled room under a 12.00-hour dark/light cycle, were allowed free access to water and laboratory chow diet and were housed for several days prior to the experiments.

Rats were injected CCl₄ intraperitoneally as a 50% (vol/vol) solution in olive oil at a dose of 2.0 mL/kg bodyweight twice a week for 12 weeks (Abe et al. 2007). Four days after the last CCl₄ injection, catheters were inserted through the jugular vein into the superior vena cava under light ether anesthesia. Then the rats were divided into two groups randomly. The rats in the valine group were administered 1.688 g/kg/day of L-valine (Ajinomoto Co., Inc., Tokyo) in lactate ringer solution through a catheter for 7 days, and the rats in the control group were administered a 10% amino acid preparation (Moriprone, Ajinomoto Co., Inc.) in lactate ringer solution to provide the same amount of nitrogen as the valine group. Seven to fifteen rats per each group were sacrificed each time on days 7, 14 and 17, after the catheters insertion (Fig. 1). The mean weights of rats were the same in two groups on each day. Blood samples were collected from the abdominal aorta. The liver was removed and cut into small pieces. Some pieces were fixed in 4% paraformaldehyde for histological analysis. Others were snap-frozen for RNA extraction.

To investigate whether the *in vivo* findings in the CCl₄-induced cirrhotic rats used in this study were similar to those of cirrhotic patients, seven rats with CCl₄-induced liver fibrosis were sacrificed and samples were collected (CCl₄ group). In parallel with the CCl₄ group, seven Wistar male rats were left untreated and served as untreated controls (untreated group).

**Peripheral blood cell counts and determination of the serum level of amino acids**

Blood cell counts were measured (Mitsubishi Chemical Medience Co.,Tokyo). Serum was prepared from blood after centrifugation at 3,000 r.p.m. for 15 minutes at 4°C and filtered. After filtra-

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**Fig. 1.** Schema of the experimental design.

Rats were treated with CCl₄ for 12 weeks or left untreated. After liver fibrosis had been established with CCl₄, administration for 12 weeks, catheters were inserted through the jugular vein into the superior vena cava under light ether anesthesia (day 0). Then the rats were divided into 2 groups (valine group and control group). The rats in valine group were administered 1.688 g/kg/day of L-valine mixed in the lactate ringer solution through a catheter for 7 days, and the rats in the control group were administered 10% amino acid preparation mixed in the lactate ringer solution to take the same amount of nitrogen. Under light ether anesthesia, the rats of two groups were sacrificed each time on days 7, 14 and 17, after catheter insertion.
tion, the supernatant solution was deproteinized with 4% sulfosalicylic acid. The concentration of amino acids was determined in a high-speed amino acid analyzer (Model L8500, Hitachi Ltd., Tokyo).

**Histological analysis of liver and bone marrow**

The liver was fixed in 4% paraformaldehyde, stained with hematoxylin and eosin (HE) stain, and Azan-Mallory (AM) stain. The extent of fibrosis was quantified using Scion Image (version alpha 4.0.3.2, Scion Corp., Frederick, MD), in three high power light microscopic fields (×100) per specimen in all rats from each group. The index of fibrosis was calculated as the area of fibrosis divided by the whole area of the microscopic field. The femoral bone marrow of rats was fixed in 10% formalin, and then stained with HE. We counted the number of megakaryocytes per high-power field (×400).

**RNA extraction and reverse transcription**

Total RNAs was extracted from rat liver using ISOGEN (Nippon Gene, Tokyo) according to the protocol supplied by the manufacturer. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA with oligo(dT)20 primer using ReverTra Ace–α- (TOYOBO CO., LTD., Osaka, Japan) as described in the manufacturer’s manual.

**Quantitative PCR analysis**

Quantitative PCR was performed using a Light Cycler Quick System 330 (Roche Diagnostics, Tokyo) with a commercially available master mix containing SYBR Green I, deoxyribonucleotide triphosphates, Mg²⁺ and TaKaRa Ex Taq HS (SYBR Permix Ex Taq, Takara Bio, Shiga, Japan). All primers were obtained from Takara Bio. Primer sequences are shown in Table 1. Amplification was carried out in a final volume of 20 µL that included 2 µL of DNA template (0.05 µg/µL), 0.4 µL of each forward and reverse primer (final concentration: 0.2 µM) and 10 µL master mix. The cycling conditions were set as follows: 55 cycles at 95°C for 5 seconds and 60°C for 20 seconds. The cycling conditions of only TGF-β1 were set as follows: 55 cycles at 95°C for 4 seconds, 58°C for 10 seconds and 72°C for 10 seconds. A calibration curve was constructed with 10-fold serial dilution of a 1.0 µg/µL cDNA from one of the samples. The relative numbers of mRNA copies was calculated as recommended by the manufacturer. The mRNA expression of ThPO, procollagen α2(I), procollagen α1(III), TGF-β1 and CTGF, was normalized using the endogenously expressed housekeeping gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control.

**Statistical analysis**

Data are presented as the mean ± s.d. Student’s t test, Welch’s t test or Mann-Whitney rank sum test was used for comparison between the groups, where appropriate. The statistically significant difference was set at p < 0.05.

**Results**

**Serum level of free amino acids**

Administration of L-valine at a dose of 1.688 g/kg/day caused about six-fold increase in serum valine compared with rats of the control group by day 7 after L-valine treatment (153.0 ± 28.6 vs. 24.5 ± 5.1 µmol/dL, p < 0.001). On day 7, the Fischer’s ratio in rats of the valine group and control group was 7.4 ± 2.1 and 1.7 ± 0.3, respectively (p < 0.001). Furthermore, on day 7, the serum level of phenylalanine (7.4 ± 1.1 vs. 10.5 ± 2.3 µmol/dL, p < 0.001), tyrosine (13.2 ± 2.9 vs. 16.2 ± 4.3 µmol/dL, p = 0.04), methionine (7.7 ± 1.3 vs. 9.0 ± 1.4 µmol/dL, p < 0.05), phosphoserine (3.4 ± 0.6 vs. 4.1 ± 0.9 µmol/dL, p = 0.03), lysine (28.2 ± 6.8 vs. 38.2 ± 8.7 µmol/dL, p < 0.01), threonine (26.7 ± 9.1 vs. 36.0 ± 9.1 µmol/dL, p = 0.01) and glycine (46.1 ± 10.6 vs. 51.7 ± 9.8 µmol/dL, p < 0.05) were lower in the valine group compared to the control group; but the serum level of glutamine was higher in the valine group (32.0 ± 4.5 vs. 28.1 ± 4.4 µmol/dL, p = 0.02). Seven days after the termination of the L-valine administration (day 14), the serum level of valine and the Fischer’s ratio in the valine group decreased to the same level observed in the control group; but the serum level of histidine was slightly increased in the control group (valine group 13.4 ± 2.3 µmol/dL vs. control group 15.6 ± 2.6 µmol/dL, p = 0.04). There were no differences between the two groups in the level of other amino acids on days 14 and 17.

**Blood cell counts**

In the rats treated with CCl₄ for 12 weeks (CCl₄ group), platelet counts were significantly higher than in untreated rats (untreated group) (108.8 ± 18.9 vs. 90.8 ± 9.3 × 10⁵/µL, p = 0.03, Table 2). Moreover, blood white cell and red cell counts of CCl₄ group were higher than those of untreated group. However, the hemoglobin concentration and hematocrit were lower in the CCl₄ group than in the untreated group (Table 2).

The platelet counts on day 7 were similar in the two groups (Table 3); on day 14 the peripheral platelet counts of the valine group were significantly higher than those of the control group (131.2 ± 38.3 vs. 106.3 ± 14.5 × 10⁵/µL, p = 0.04, Table 3). The blood white cell counts, red cell counts, hemoglobin concentration and hematocrit did not differ sig-

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significantly between the two groups during the experiment.

**Bone marrow megakaryocytes**

To investigate in vivo thrombopoiesis, bone marrow megakaryocytes were counted (Fig. 2). The bone marrow thrombopoiesis was significantly suppressed in the CCl$_4$ induced cirrhotic rats (CCl$_4$ group) (Fig. 2, left columns). The number of megakaryocytes in the CCl$_4$ group was significantly lower than that in the untreated group (11.6 ± 3.7 vs. 17.4 ± 2.2 per field, $p < 0.05$). We next analyzed the effect of L-valine, showing that L-valine administration ameliorated the decrease in the bone marrow thrombopoiesis (Fig. 2, right columns). While there was no differences between the control group and CCl$_4$ group in the number of megakaryocytes, the number of megakaryocytes of the valine group was significantly higher than that of the control group on day 14 (18.0 ± 2.1 vs. 13.5 ± 2.2 per field, $p < 0.01$). Thus, L-valine administration restored the bone marrow thrombopoiesis to the level of untreated rats.

**Thrombopoietin mRNA expression in the liver of rats with carbon tetrachloride-induced cirrhosis and effects of L-valine administration**

The expression level of ThPO mRNA was significantly lower in the liver of rats treated with CCl$_4$ than in the untreated group ($p = 0.04$, Fig. 3A). Subsequently, ThPO mRNA levels in the liver of CCl$_4$-treated rats, valine-treated cirrhotic rats and control group were measured by real-time RT-PCR (Fig. 3B,C). ThPO mRNA expression in the valine group was significantly higher than that in the control group on day 14, when the peripheral platelet counts were increased (1.37-fold, $p = 0.03$, Fig. 3C). However, there were no differences between the two groups in ThPO mRNA expression in the liver on day 7 (Fig. 3B).

**Histopathological analysis of the liver**

The liver from the untreated group showed no fibrosis. On the other hand, hepatic fibrosis was evident in the liver of CCl$_4$-treated rats. On day 7, the liver of control and valine-treated rats showed bridging fibrosis and nodule formation (Fig. 4A, B). Although thick bridging fibrosis and

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Plt; blood platelet counts, WBC; blood white cell counts, RBC; blood red cell counts, Hb; hemoglobin concentration, and Ht; hematocrit.

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Day 14

|            | Control group | Valine group | $p$   |
| Plt ($\times 10^4/\mu l$) | 106.3 ± 14.5 | 131.2 ± 38.3 | 0.04* |
| WBC ($\times 10^3/\mu l$) | 19.7 ± 3.7 | 21.7 ± 5.0 | 0.28 |
| RBC ($\times 10^5/\mu l$) | 757.5 ± 88.1 | 706.9 ± 97.8 | 0.19 |
| Hb (g/dl) | 13.6 ± 1.2 | 12.8 ± 1.8 | 0.20 |
| Ht (%) | 40.2 ± 3.6 | 37.9 ± 4.8 | 0.29 |

Plt; blood platelet counts, WBC; blood white cell counts, RBC; blood red cell counts, Hb; hemoglobin concentration, and Ht; hematocrit. *, statistically significant difference.
nodules were also found in the liver of the control group on day 14, thinner fibrosis was observed only in the valine group (Fig. 4C, D). Furthermore, the livers from the valine group showed no nodules, whereas the control group presented nodules on day 17 (Fig. 4E, F). Quantitative analysis showed that the mean fibrotic areas in the liver improved...
Fig. 4. Effect of L-valine on the CCl₄-induced liver fibrosis.

Shown are representative photomicrographs of the liver histology of the control and valine group rats (Azan-Mallory staining, × 100): A, control group, day 7; B, valine group, day 7; C, control group, day 14; D, valine group, day 14; E, control group, day 17; and F, valine group, day 17. On day 7, the liver of two groups showed bridging fibrosis and nodule formation (A, B). Although thick bridging fibrosis and nodules were found in the liver of the control group on day 14 (C, white arrows), thinner fibrosis was observed only in the valine group (D, black arrows). In the valine group the livers showed no nodules (F, black arrows) while the control group presented nodules on day 17 (E, white arrows). G. Index of fibrosis. The mean fibrotic areas in the valine group were smaller than those in the control group on days 14 and 17 (p < 0.001). The animals used were untreated group (n = 7), control groups, day 7 (n = 15), day 14 (n = 11) and day 17 (n = 7), and valine groups, day 7 (n = 13), day 14 (n = 14), and day 17 (n = 8).

Fig. 5. Effect of CCl₄ on the expression of procollagen, TGF-β1 and CTGF mRNAs.

Rats were treated with CCl₄ for 12 weeks (n = 7) or left untreated (n = 7). The expression of mRNA in the livers was assessed by real-time RT-PCR, and is expressed as the fold-induction relative to the liver of untreated group. Shown are the relative expression levels of (A) procollagen α2(I) (ProCol I), (B) procollagen α1(III) (ProCol III), (C) TGF-β1, and (D) CTGF mRNAs. In the CCl₄ group, the expression levels of procollagen α2(I), procollagen α1(III), TGF-β1 and CTGF mRNAs were significantly higher than those in the untreated group by 2.13-fold (p = 0.0001), 1.27-fold (p = 0.01), 1.37-fold (p = 0.009), and 2.20-fold (p < 0.05), respectively.
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with time and those of the valine group were smaller than the fibrotic areas found in the control group on days 14 and 17 (p < 0.001, Fig. 4G).

Effects of L-valine on the mRNA expression in the liver of factors associated with hepatic fibrosis

To investigate the molecular basis of the improvement of hepatic fibrosis after treatment with L-valine, we measured the expression levels of procollagen \( \alpha_2(I) \), procollagen \( \alpha_1(III) \), TGF-\( \beta \)1 and CTGF mRNAs. In the CCl\(_4\) group, the expression of procollagen \( \alpha_2(I) \), procollagen \( \alpha_1(III) \), TGF-\( \beta \)1 and CTGF mRNAs increased significantly compared to the untreated group (2.13-fold, \( p = 0.0001 \); 1.27-fold, \( p = 0.01 \); 1.37-fold, \( p = 0.009 \); and 2.20-fold, \( p < 0.05 \), respectively, Fig. 5A-D). On the other hand, in the valine group, the expression levels of procollagen \( \alpha_1(III) \), TGF-\( \beta \)1 and CTGF mRNAs were significantly decreased on day 17 compared to the control group (0.78-fold, \( p < 0.05 \); 0.51-fold, \( p = 0.03 \); and 0.59-fold, \( p = 0.04 \), respectively, Fig. 6A-C). The expression level of procollagen \( \alpha_2(I) \) mRNA tended to decrease in the valine group compared to the control group, but the difference was not statistically significant (0.75-fold, \( p < 0.1 \)).

Discussion

In this study, we demonstrated that L-valine increased hepatic ThPO mRNA level, bone marrow megakaryocyte counts and blood platelet counts in rats with CCl\(_4\)-induced liver fibrosis. Martin et al. (1997) found that the level of ThPO mRNA was decreased in the liver of cirrhotic patients. The cirrhotic rats used in the present study showed the histological findings and the decrease of ThPO mRNA level similar to those observed in cirrhotic patients. However, peripheral blood cell counts were greater in CCl\(_4\)-treated rats compared to untreated rats. This might be explained by the dehydration caused by ascites and decrease in food and water intake. Despite the decreased hematocrit and hemoglobin, red cell counts were increased in CCl\(_4\)-treated rats. Therefore, it is conceivable that CCl\(_4\)-treated rats showed hypochromic microcytic anemia and dehydration. Although no thrombocytopenia was observed in peripheral blood, the count of megakaryocytes in bone marrow was decreased. These findings indicate that thrombopoiesis was suppressed in CCl\(_4\)-treated rats. Our results raise the possibility of a thrombopoietic effect of L-valine in patients with liver diseases.

When recombinant ThPO was administered to normal mice for 5 days, the peripheral platelet count reached its maximum level 7 days after the onset of ThPO administration (Kabaya et al. 1996). In the present study, ThPO mRNA expression, as well as peripheral platelet and bone marrow megakaryocyte counts were significantly increased...
in the cirrhotic rats 14 days after the beginning of continuous L-valine administration. The time course of the L-valine-induced increase of platelet and megakaryocyte counts in our experiments seemed to be longer than that observed with recombinant ThPO (Kabaya et al. 1996). It is unclear why the thrombopoietic effects were observed on day 14, but not on day 7, the day of the termination of L-valine. L-valine treatment is successful in shortening the time course of recovery from CCl\textsubscript{4}-induce liver injury and thus has shown thrombopoietic effect 7 days after the termination of L-valine treatment.

In this study, L-valine reduced hepatic fibrosis in CCl\textsubscript{4}-treated rats. Procollagen \(\alpha2(1)\), procollagen \(\alpha1(III)\), TGF-\(\beta1\) and CTGF are closely associated with liver fibrosis, and antiﬁbrotic agents such as HGF have been shown to exert their effect through the suppression of those mRNA levels (Ignotz et al. 1987; Raghch et al. 1987; Yasuda et al. 1996). We observed that those mRNA levels were similar in the control and valine groups on days 7 and 14. On day 7, there were no differences in liver fibrosis between the two groups. On day 14, however, the livers of rats in the valine group showed thinner bridging fibrosis and only a few nodules. On day 17, there were no nodules. At the same time, the mRNA levels of procollagen \(\alpha1(III)\), TGF-\(\beta1\) and CTGF were signiﬁcantly suppressed in the livers of the valine group. It is tempting to speculate that L-valine might exert an antiﬁbrotic effect by reducing the TGF-\(\beta1\) mRNA level, because TGF-\(\beta1\) is strongly associated with hepatic fibrosis and induces collagen formation by increasing CTGF (Paradis et al. 1999) and procollagen mRNA (Ignotz et al. 1987; Raghch et al. 1987). However, signiﬁcant reduction of liver fibrosis by L-valine was shown on day 14 when the mRNA levels of factors associated with liver fibrosis were similar in the control and valine group. This might be explained by the possibility that L-valine improved the recover from CCl\textsubscript{4}-induce liver injury by promoting liver regeneration as a substrate for the production of proteins. Furthermore, a recent study showed that platelets reduce liver fibrosis in mice treated with CCl\textsubscript{4} (Watanabe et al. 2009). It is conceivable that platelets may contribute to the reduction of liver fibrosis in the present study, because thrombopoietic effect was observed on day 14.

BCAAs are essential for protein anabolism. Several studies have reported long-term supplementation with BCAAs to treat protein malnutrition in patients with decompensated cirrhosis, and that this therapy improved or maintained the nutritional status, as well as increased the survival rates (Ghanta et al. 2005; Muto et al. 2005). In this study, L-valine administration was associated with the decreased serum level of several amino acids such as phenylalanine, tyrosine, methionine, phosphoserine, lysine, threonine and glycine. It is likely that the decreased amino acid metabolism by liver injury is improved in early stage by the L-valine administration. L-valine might have improved the liver disease by promoting protein synthesis and energy production. Thus, L-valine might have ameliorated the thrombopoietin production of hepatocytes and liver fibrosis by promoting the recovery from liver injury.

It has been reported that leucine stimulates HGF production by hepatic stellate cells through mammalian target of rapamycin pathway (Tomiya et al. 2007). The beneficial effects observed in patients with cirrhosis during BCAAs supplementation might stem from an increased liver regeneration, compensating for progressive liver-cell death. Furthermore, it has been demonstrated that an increased concentration of valine could lead to a recovery of the impaired function of dendritic cells in cirrhotic patients (Kakazu et al. 2007). Several BCAAs’ effects on immune function provide the rationale of BCAAs therapy for patients with cirrhosis (Calder 2006). However, more research is needed to understand the mechanism that BCAAs exert beneﬁcial effects in cirrhotic patients.

In conclusion, our data support the rationale for a BCAAs therapy for cirrhotic patients.

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References


