Anti-fibrotic effects of branched-chain amino acids

on hepatic stellate cells

Running title: The effect of branched-chain amino acids

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Abstract

Background/Aims: Patients with liver cirrhosis (LC) have low levels of branched-chain amino acids (BCAAs). There is accumulating evidence that BCAAs have anti-fibrotic effects in cirrhosis. This study is aimed to evaluate the effect of BCAAs on the function and phenotype of activated hepatic stellate cells (HSCs).

Methods: LX-2, an immortalized human stellate cell line, was used in in vitro experiments. LX-2 cells were exposed to TGF-β1 and BCAAs or to valine, leucine, and isoleucine, which are components of BCAAs. Activation of the TGF-β signaling pathway in LX-2 cells was observed using real-time quantitative PCR and western blotting.

Results: The increased expression of SNAI1 was observed in LX-2 cells activated by TGF-β1. After BCAA treatment, its expression was significantly decreased at the mRNA level. The increased expression of Col1α1 and TIMP2 at the mRNA level and α-SMA at the protein level in activated LX-2 cells decreased after BCAA treatment. Among the BCAA components, leucine and valine significantly abrogated TGF-β-induced activation of LX-2 cells. BCAA treatment led to the decreased phosphorylation of Smad2 and p38 proteins, which are markers for Smad and Smad-independent p38 MAPK signaling pathways, respectively.

Conclusions: BCAA treatment can improve hepatic fibrosis by directly affecting the activated state of hepatic stellate cells through inhibition of the TGF-β signaling pathway. Among BCAA components, leucine and valine mainly abrogated TGF-β-induced activation of HSCs. Our results suggest that BCAA may be used to attenuate the progression of liver fibrosis.

Keywords: branched-chain amino acids, hepatic stellate cells, fibrosis
Introduction

Liver cirrhosis (LC) is a disease of global concern, responsible for more than one million worldwide deaths in 2010 [1]. Although LC was previously considered to be irreversible, there is overwhelming evidence that regression of fibrosis is possible with therapy that targets specific causes of cirrhosis, such as potent anti-viral therapies for viral hepatitis and abstinence of alcohol. In addition, many researchers have focused on the process of fibrogenesis to develop anti-fibrotic therapies.

Hepatic fibrosis means the accumulation process of extracellular matrix (ECM) proteins, and hepatic stellate cells (HSCs), which reside in the space of Disse, are the main producers of ECM proteins. Following liver injury, HSCs undergo transdifferentiation from quiescent cells into fibrogenic, proliferative, and contractile myofibroblast-like cells, characterized by expression of α-SMA [2]. Transforming growth factor-β (TGF-β), the most predominant fibrogenic cytokine, is secreted from several cells including HSCs in the liver during disease conditions and activates HSCs to produce ECM proteins including type I and III collagen and fibronectin, leading to hepatic fibrosis [3]. Thus, controlling HSC activation is considered a promising target of anti-fibrotic therapies.

 Branched-chain amino acids (BCAAs) are composed of valine, leucine, and isoleucine and are essential for synthesis of body proteins while also exerting inhibitory effects on protein degradation [4]. In LC patients, the serum ratio of BCAAs to aromatic amino acids (AAAs) is characteristically low under several conditions, such as detoxification of ammonia in skeletal muscle or insufficient nutritional intake. Therefore, previous researchers tried to identify whether BCAA supplementation could improve the prognosis of LC patients. Supplementation with BCAAs had beneficial effects on hepatic encephalopathy without
adverse events, mainly by enhancing the ratio of BCAAs to AAAs. Thus, recent guidelines recommend oral administration of BCAAs for such patients.

In addition, it has been suggested that BCAA supplementation had beneficial effects including improvement of insulin sensitivity, regression of fibrosis, and reduced incidence of hepatocellular carcinoma (HCC) in LC patients [5,6]. In a previous study, we showed that BCAA treatment produced anti-cancer and anti-fibrotic effects in a rat model with LC and HCC induced by diethylnitrosamine. The BCAA-treated group showed down-regulation of fibrosis markers, such as TGF-β1 and collagen, at both mRNA and protein levels. Expression of angiogenesis and apoptosis inhibitor markers also decreased with BCAA treatment, which could lead to suppression of HCC development [7].

However, most of the studies that demonstrated a protective effect of BCAAs against hepatic fibrosis were population-based cohorts or in vivo experiments. Thus, it is unclear as to the detailed mechanism of BCAA treatment in the process of fibrogenesis. As HSCs are central to the process of hepatic fibrosis, we reasoned that BCAAs would have a role in controlling activation of HSCs. To address this, we used LX-2 cells, a human HSC line, and evaluated the direct role of BCAAs in LX-2 cells. We also analyzed the individual effect of each component of BCAAs in LX-2 cells to determine which had the strongest effect on fibrosis regression.

**Methods**

**Chemicals and reagents.** Recombinant human TGF-β1 (240-B) was purchased from R&D
Systems (Minneapolis, MN, USA). The BCAA components (valine, leucine, and isoleucine) and amino acid-free medium (ZERO medium) were obtained from Ajinomoto Pharmaceuticals Co. (Tokyo, Japan; Table 1). BCAA mixture (Leu/Ile/Val = 2:1:1.2) was freshly dissolved in nuclease-free water (Ambion, AM9930) at 100 mmol/L.

**Cell culture and treatment.** Human HSCs (LX-2; MilliporeSigma, Burlington, MA, USA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic. Low-amino acid medium (× 1/2 DMEM) was prepared by diluting DMEM with ZERO medium. We evaluated the effects of BCAAs on LX-2 cells cultured in low-amino acid medium to create an environment for LX-2 cells similar to that of HSCs in a cirrhotic liver. After incubation for 24 hours, LX-2 cells were transferred to low-amino acid medium and starved for 24 hours. Then, the cells were treated with different concentration (5, 10 and 20 mM) of BCAAs or each component of BCAAs, leucine, isoleucine and valine for 1 hour before the addition of TGF-β1 (2.5 ng/mL). After incubation for 48h, the cells were harvested for analysis.

**RNA extraction, real-time quantitative PCR and semi-quantitative RT-PCR.** Total RNA was extracted using Trizol reagent (Thermo Fisher Scientific, Basingstoke, UK), and 1 μg of total RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Real-time quantitative PCR was performed using a Light Cycler® 480 II (Roche Diagnostics, Mannheim, Germany) with the Light Cycler® 480 Probes Master Reaction Mix (Roche). PCR reactions were performed with the TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, USA). The levels of target gene mRNA transcripts relative to control GAPDH were determined by 2−ΔΔCt. PCR amplifications were
performed at 95 °C for 15 seconds and subjected to 55 cycles of 95 °C for 10 seconds, 60 °C for 30 seconds, and 72 °C for 1 second. The following TaqMan probes were used: Col1α1, SNAI1, SNAI2, and GAPDH (Table 2). Semi-quantitative RT-PCR amplifications were performed for TIMP1 and TIMP2 at 94 °C for 5 minutes and subjected to 23–36 cycles of 94 °C for 30 seconds, 56–60 °C for 30 seconds, 72 °C for 1 minute and a final extension for 5 minutes at 72 °C. The primer sequences were as follows (5’ → 3’): TIMP1, 5’–AGACCTACACTGTTGGCTGTGAG–3’ and 5’–GACTGGAAGCCCTTTTCAGAG–3’; TIMP2, 5’–ATGCACATCACCCCTCTGTGA–3’ and 5’–CTCTGTGACCAGATCCCATCC–3’; GAPDH, 5’–GAGTCAACGGATTTGGTCTCGT–3’ and 5’–TGATTTCTCGAGGATCT–3’.

Western blotting analysis. Equivalent amounts of protein lysates (10 μg/lane) were separated by 10 % SDS-polyacrylamide gel electrophoresis (PAGE) and subjected to western blot analysis with primary antibodies for α-SMA (Sigma-Aldrich, A2547), p-p38 (Cell Signaling Technology, #9211), p38 (Cell Signaling Technology, #9212), p-Smad2 (Cell Signaling Technology, #8828) and Smad2 (Cell Signaling Technology, #8685). Antibodies to α-tubulin (Sigma-Aldrich, T5168) served as loading controls.

Statistical analysis. Unpaired t-tests or repeated measures ANOVA were used for statistical analysis. A two-sided $P$ value < 0.05 was considered statistically significant. Data was analyzed using SPSS 20.0 (IBM Co., Armonk, NY, USA).
Results

The effects of BCAAs on the activation of LX-2 cells cultured in low-amino acid medium. We evaluated the effects of BCAAs on LX-2 cells cultured in low-amino acid medium (Fig. 1). The LX-2 cells activated by TGF-β1 (2.5 ng/mL) adopted a flattened morphology (Fig. 1a). Induction of SNAI1 and SNAI2 expression is mediated by the TGF-β1/Smad signaling pathway [8,9], and expression of α-SMA, Col1α1, TIMP1, and TIMP2 is increased in activated HSC. Following treatment with 20 mM BCAA, the increased mRNA expression of SNAI1 on activated LX-2 cells significantly decreased ($P = 0.036$). Expression of Col1α1 and TIMP2 at the mRNA level decreased with 20 mM BCAA ($P = 0.056$ for both Col1α1 and TIMP2) (Fig. 1b-c). Western blotting showed that α-SMA expression increased with TGF-β1 (2.5 ng/mL) and decreased with BCAA treatments at 5, 10, and 20 mM without statistical significance ($P = 0.183$ for 5 mM BCAA, $P = 0.098$ for 10 mM and $P = 0.136$ for 20 mM compared to the level in activated LX-2 cells without BCAA) (Fig. 1c). Unpaired t-tests were used to assess for statistical difference.

The effect of each component of BCAA on the activation of LX-2 cells. We treated LX-2 cells that had been cultured in low-amino acid medium with valine, leucine and isoleucine at different concentrations (5, 10 and 20 mM) (Fig. 2). The increased expression of SNAI2 at the mRNA levels on activated LX-2 cells decreased with 20 mM leucine ($P = 0.051$) (Fig. 2a) and the increased expression of Col1α1 decreased with 20 mM valine ($P = 0.083$) (Fig. 2c). Protein expression of α-SMA in LX-2 cells increased with TGF-β1 (2.5 ng/mL) treatment and significantly decreased with 20 mM leucine treatment ($P = 0.033$ compared to the level in activated LX-2 cells without leucine) (Fig. 2b). Similarly, the increased protein expression of
α-SMA with TGF-β1 (2.5 ng/mL) in LX-2 cells significantly decreased with 20 mM valine treatment ($P = 0.024$ compared to the level in activated LX-2 cells without valine) (Fig. 2d). There was no significant change in expression markers in activated LX-2 cells treated with isoleucine (Fig. 2e-f). Unpaired t-tests were used to assess for statistical difference.

The expression of markers in the TGF-β signaling pathway. To investigate the role of BCAAs in the TGF-β signaling pathway of LX-2 cells, we analyzed the expression of molecular markers Smad2 and p38, which are involved in Smad and Smad-independent p38 MAPK pathways, respectively (Fig. 3). Western blotting showed that the increased phosphorylation of Smad2 in the Smad pathway by TGF-β1 (2.5 ng/mL) stimulation decreased after BCAA treatment in LX-2 cells ($P = 0.032$ with BCAAs of 5, 10, and 20 mM and $P = 0.013$ with BCAAs of 5 and 10 mM). The increased protein expression of p38 phosphorylation by TGF-β1 (2.5 ng/mL) in the p38 MAPK pathway also decreased by BCAA treatment ($P = 0.008$ with BCAA of 5, 10 and 20 mM and $P = 0.078$ with BCAA of 5 and 10 mM). Repeated measures ANOVA was used to assess for statistical difference.

Discussion

As several therapeutic approaches have been investigated to reverse cirrhosis, the role of HSCs, the major contributors to hepatic fibrosis, has been in the spotlight. Based on the recent results that BCAA supplementation can improve the prognosis of LC patients by inhibiting progression of cirrhosis, we focused on HSCs and studied whether BCAAs could
reduce activation of HSCs. In this study, we identified that BCAAs exerted anti-fibrotic effects directly on HSCs. Leucine and valine, among the three components of BCAAs, showed inhibitory effects on activated LX-2 cells by TGF-β treatment. In addition, this study showed that BCAA treatment down-regulated expression of markers involved in Smad pathway and Smad-independent p38 MAPK pathway of TGF-β signaling in HSCs.

The positive effects of BCAA treatment in LC patients have been identified in various clinical aspects. In addition to the well-known effects of BCAAs on hepatic encephalopathy, recent studies demonstrated that BCAAs improved liver function in LC patients. A multicenter study with two-year BCAA treatment in LC patients with Child–Turcotte–Pugh (CTP) class B showed that Model for End-Stage Liver Disease score, CTP score, and bilirubin level were significantly improved, and similar results were obtained from an Italian randomized controlled trial in which serum bilirubin level and CTP score were improved [10,11]. LC patients supplemented with long-term oral BCAA had a lower incidence of major cirrhosis-related events than control group in a prospective study [12]. Collectively, these results suggest that BCAA treatment improves hepatic reserve function and attenuates fibrosis process.

In this study, we tried to elucidate the detailed mechanism of BCAA effects on HSC activation in hepatic fibrosis. Expression markers such as α-SMA, Col1α1, SNAI1, and SNAI2, indicating LX-2 cell activation, significantly increased with TGF-β1 stimulation and decreased with BCAA treatment. We previously identified down-regulation of fibrosis markers including α-SMA, Col1α2, and Col3α1 at the protein and mRNA levels following BCAA treatment in rat liver tissue with induced fibrosis. Through this study, we demonstrated that BCAA directly inhibited activation of HSC. This is consistent with previous results that gene expression of TGF-β1 and Smad involved in the signaling pathway
of HSC activation was significantly changed toward attenuating hepatic fibrosis by BCAA treatment, and the number of activated HSCs with expression of α-SMA clustered around fibrous septa decreased with BCAA treatment in rat liver tissue with CCl₄-induced fibrosis [6]. Another study with LX-2 cells activated by TGF-β1 stimulation demonstrated that gene expression of markers related to the TGF-β1 signaling pathway, such as TGFβR1, p-Smad3, p-Smad3L, and α-SMA, significantly decreased with BCAA treatment [13].

The downstream pathway of TGF-β signaling to induce fibrosis is complex. TGF-β directly acts on HSCs and binds to TGF-β receptor 2, which subsequently activates TGF-β receptor 1 (TGFR1). This activated receptor phosphorylates Smad2 and Smad3 proteins, and phosphorylated Smad2/3 forms a complex with Smad4 to regulate transcription of ECM genes, such as collagens, plasminogen activator inhibitor-1, and fibronectin in the nucleus [3,14]. In this study, we showed that expression of phosphorylated Smad2 increased with TGF-β1 treatment and significantly decreased with BCAA treatment in LX-2 cells, suggesting that the inhibitory effect of BCAAs acts on the stage before Smad2 is phosphorylated.

Aside from this canonical Smad pathway, activated TGFR1 induces other signal transducers, such as the JNK and p38 MAPK pathway, the PI3K-AKT pathway, and Rho family GTPases, collectively referred to as Smad-independent pathways [15-17]. In this study, increased expression of p38 phosphorylation by TGF-β stimulation was suppressed by BCAA treatment in LX-2 cells, indicating that BCAA could inhibit HSC activation through both Smad and Smad-independent p38 MAPK signaling pathways. Decreased expression of p38 phosphorylation by BCAA treatment was demonstrated in our previous study and was associated with suppression of tumor angiogenesis [7]. Activation of the p38 MAPK signaling pathway is associated with regulation of gene expression of transcription factors,
cell surface receptors and cytokines, and the EMT process in hepatocytes [18,19]. Thus, it is speculated that the effect of BCAAs through inhibition of the p38 MAPK pathway is not limited to attenuating hepatic fibrosis by inhibiting HSC activation.

BCAAs is well-known activator for mammalian target of rapamycin complex 1 (mTORC1) and leucine is known to be closely associated with mTOR activation [20,21]. It was suggested that the effect of BCAAs in TGF-β signaling pathway of HSC was through activation of mTORC1, and the effect of leucine, especially among the BCAA components, was suggested as attenuation of fibrosis of skeletal muscle in rat. In muscle tissue of rats administered leucine, expression of phosphorylated TGFR1 and Smad2/3-positive nuclei decreased compared to control groups [13,22]. In this study, although the effect of leucine was not remarkable, we identified that leucine and valine, except for isoleucine, reduced LX-2 cell activation.

This study has some limitations. We focused on investigating the mechanism of BCAA effect on HSC activation and used the human HSC cell line LX-2, widely used in culture-based studies for hepatic fibrosis. However, it is not enough to explain the effect of BCAA on HSC based only on the results from LX-2 cells. Second, this study identified decreased expression of phosphorylation of Smad2 proteins and p38 after BCAA treatment in the TGF-β signaling pathway in HSC, but we did not identify expression of other genes involved in different stages of Smad and Smad-independent pathways. Because of this, it was not possible to determine the stage inhibited by BCAAs in the TGF-β signaling pathway. Further study is needed to elucidate the precise mechanism of BCAAs in the process of hepatic fibrosis, which will also advance future research into development of anti-fibrotic therapies.

In conclusion, this study demonstrated that BCAA treatment could be beneficial for reducing hepatic fibrosis by direct action on HSCs to affect both Smad and Smad-independent p38
MAPK signaling pathways in TGF-β signaling in HSC. Among the BCAA components, leucine and valine showed an effect of anti-fibrosis. Through the findings of this study, we expect BCAAs to have a role in development of new anti-fibrosis therapies.
Key message

• BCAA treatment inhibited TGF-β-induced activation of LX-2 cells.

• Among the BCAA components, leucine and valine inhibited activated LX-2 cells.

• BCAA treatment reduced Smad2 and p38 phosphorylation, which are involved in the downstream pathways of TGF-β1 signaling.
Acknowledgements

Author contributions

Guarantor of the article: Si Hyun Bae

Study concept and design: Hae Lim Lee, Jungmin Lee and Si Hyun Bae; collecting and interpreting data: Hae Lim Lee, Jungmin Lee and Si Hyun Bae; Contributed reagents/materials/analysis tools: Hae Lim Lee, Jung Hoon Cha, Sungwoo Cho, Pil Soo Sung, Wonhee Hur, Seung Kew Yoon, Jungmin Lee, Si Hyun Bae; Wrote the paper: Hae Lim Lee, Jungmin Lee and Si Hyun Bae. All authors have approved this final version of the manuscript.

Competing interests

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Financial support: The authors declare no financial conflicts of interest.

Declaration of personal interests: None
References


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Table 2. List of Taqman primers used for quantitative real-time PCR

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**Figure legends**

Figure 1. Effects of BCAA treatment on activated LX-2 cells cultured in low-amino acid medium. Activated LX-2 cells were treated with 5, 10 and 20 mM of BCAA. (a) Morphological changes of LX-2 cells were observed under a microscope (100 × magnification). (b-c) Real-time quantitative PCR of Col1α1, SNAI1, SNAI2, TIMP1 and TIMP2 was performed in LX-2 cells. Bar graphs represent the means ± s.e.m. (n = 3). Unpaired t-tests were performed in LX-2 cells. (d) Western blotting of α-SMA was performed in LX-2 cells. Bar graphs represent the means ± s.e.m. (n = 3). Unpaired t-tests were performed in LX-2 cells.

Figure 2. Effects of individual treatments with leucine, valine and isoleucine among the BCAA components, on LX-2 cells activated by TGF-β1 treatment. LX-2 cells were cultured in low-amino acid medium. Activated LX-2 cells were treated with 5, 10, and 20 mM of BCAA. Bar graphs represent the means ± s.e.m. (n = 3). Unpaired t-tests were performed. (a) Real-time quantitative PCR of Col1α1, SNAI1, and SNAI2 was performed in LX-2 cells treated with leucine. (b) Western blotting of α-SMA was performed in LX-2 cells treated with leucine. (c) Real-time quantitative PCR of Col1α1, SNAI1, and SNAI2 was performed in LX-2 cells treated with valine. (d) Western blotting of α-SMA was performed in LX-2 cells treated with valine. (e) Real-time quantitative PCR of Col1α1, SNAI1, and SNAI2 was performed in LX-2 cells treated with isoleucine. (d) Western blotting of α-SMA was performed in LX-2 cells treated with isoleucine.
Figure 3. Change in expression of Smad2 and p38 phosphorylation in LX-2 cells activated by TGF-β treatment. LX-2 cells were cultured in low-amino acid medium. Activated LX-2 cells were treated with 5, 10, and 20 mM of BCAA. (a) Western blotting was performed against p-Smad2 in LX-2 cells. Means ± s.e.m. are shown (n = 3). Repeated measures ANOVA tests were performed. (b) Western blotting was performed against p-p38 in LX-2 cells. Means ± s.e.m. are shown (n = 3). Repeated measures ANOVA tests were performed.
**Figure 3**

A. Western blot analysis showing the effect of BCAA (mM) and TGF-β1 (ng/mL) on p-Smad2, Smad2, and α-tubulin expression.

B. Western blot analysis showing the effect of BCAA (mM) and TGF-β1 (ng/mL) on p-p38, p38, and α-tubulin expression.

Graphs depicting the relative p-Smad2 and p-p38 protein expression with statistical significance indicated by *p < 0.05.