Effects of Long-Term Oral Supplementation with Branched-Chain Amino Acids for Spontaneous Development of Hepatocellular Carcinoma in Long-Evans Cinnamon Rats

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Abstract

Branched-chain amino acids (BCAAs) are often used for nutritional support in patients with decompensated liver cirrhosis. However, the effect of long-term oral supplementation with BCAAs on the onset and progression of hepatocellular carcinoma (HCC) in patients with chronic liver disease is unknown. We investigated the effect of long-term oral supplementation with BCAAs for a model of spontaneous development HCC in Long-Evans Cinnamon (LEC) rats. The BCAA group (6 rats) and control group rats (6 rats), which were fed BCAA diet or control diet, were observed up to 12 months of age from at 6 months of age. There were no significant differences in serum albumin or liver function values between the two groups. Serum copper concentrations and transferrin levels were higher in the BCAA group than in the control group (p < 0.05). Fischer's ratio was higher in the BCAA group than in the control group, but the difference was not significant. The nodular lesions with HCC were observed on the liver surface in all rats in both groups. The severity of the nodular findings was moderate and severe in each of two rats in the control group, but only minimal or mild in all rats in the BCAA group. Histologically, BCAA group had less extensive tumor nodule formation per unit area and mild necroinflammatory changes in non-tumor areas. In the BCAA group, staining degree for iron and copper were mild in all rats compared to the control group. Long-term oral supplementation with BCAAs in LEC rats stimulated the release of copper into blood and inhibited the deposition of copper into hepatic tissue. BCAAs did not cause carcinogenesis, tumor growth, or tumor progression in LEC rats.

Key Words:
Branched-Chain Amino Acid, Hepatocellular Carcinoma, Long-Evans Cinnamon Rat

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1. Introduction

Branched-chain amino acids (BCAAs) are often used for nutritional support in patients with decompensated liver cirrhosis\(^1\)\(^2\). We have already reported that long-term oral supplementation with BCAAs helps to maintain serum albumin levels in patients with compensated cirrhosis, where protein synthesis is relatively well preserved\(^3\). The most potentially serious complication in patients with compensated cirrhosis is the development of hepatocellular carcinoma (HCC). However, the effect of long-term oral supplementation with BCAAs on the onset and progression of hepatocellular carcinoma in patients with chronic liver disease is unknown. We therefore investigated the effect of long-term oral supplementation with BCAAs for a model of spontaneous development HCC in Long-Evans Cinnamon rats.

2. Materials and Methods

1) Animals

Twenty male Long-Evans Cinnamon (LEC) rats at 10 weeks of age were purchased from Charles River Japan (Kanagawa, Japan; Line name, Crj:LEC). Rats had free access to a standard diet (Clea Japan, Tokyo, Japan; copper content, 9.2 g/kg) and distilled water. The 12 rats that survived to 6 months of age were divided into the BCAA group (6 rats) and the control group (6 rats).

Starting at 6 months of age, the rats were housed in a controlled environment (room temperature, 22 to 23 °C; humidity, 50 to 60 %; lighting, 12 hours daily; ventilation rate, 15 exchanges per hour; sound level, 50 phones) to 12 months of age. Two groups of six rats each were fed either the control diet containing 217 g/kg casein or BCAAs diet containing 200 g/kg casein plus 25 g/kg of BCAAs (valine/leucine/isoleucine, 1.2:2:1 weight ratio; Ajinomoto Co., Tokyo, Japan) (Table-1). Rats were given free access to the experimental diet and drinking water. Rats were weighed weekly.

2) Methods

The BCAA group and control group rats were observed up to 12 months of age for survival and body weight changes. After a 24-hour fasting period, the laparotomy was performed under diethyl ether anesthesia. Blood for biochemical and amino acid analysis was collected from the inferior vena cava. After the rats were sufficiently bled, the liver was resected, visually inspected for the presence and number of tumor nodules, and submitted for histological examination. Amino acid analysis of the liver tissue was performed as follows. Liver tissue (1g) was carefully weighed, isotonic phosphate-buffered saline (PBS) was added, and a 20 % homogenate (ice cold) was prepared with a Polytron PT3100 homogenizer (Kinematic Inc. Cincinnati OH). Sulfsalicylic acid solution was added to the homogenate, the sample was centrifuged at 10 000 rpm at 4 °C for 20 min, deproteinated, and assayed with an amino acid analyzer.

3) Statistics

Values were expressed as mean ± SD, and differences in mean values were tested by the one-way analysis of variance followed by the Student's test.

3. Results

1) Body weight changes

Body weight at the start of the study (6 months of age) was 262 ± 29 g in the control group and 261 ± 28 g in the BCAA group. Between 6 to 7 months of age, there
was no significant difference between the groups. At 7 months of age, the BCAA group tended to weigh more, but the difference was not significant. At all subsequent weekly measurements to 12 months of age, the BCAA group was slightly heavier (Fig.1). No rats in either group died during the observation period.

2) Body weight and liver weight at time of sacrifice

At the time of sacrifice, body and liver weights of the 12-month-old rats in the BCAA group (336 ± 14 g, 9.0 ± 0.5 g, respectively) were slightly higher than in the control group (324 ± 14 g, 8.7 ± 0.7 g, respectively), but the differences were not significant. The liver/body weight ratio in both groups was about 2.7%.

3) Visual Inspection of liver surface for tumor nodules

Grayish white, hard small HCC nodules were observed on the liver surface in all rats in both groups.

<table>
<thead>
<tr>
<th>Table 2. Macrosopic Findings of Tumor Nodules on Liver Surface</th>
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<tbody>
<tr>
<td>Group (n)</td>
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<tr>
<td>Control (6)</td>
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<td>BCAA (6)</td>
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Based on the nodule occupancy rate, minimal: 20%; mild: 20–40%; moderate: 40–60%; severe: 60%.

Fig. 1. Body weight changes show that the BCAA group was slightly heavier, but no significant difference between the groups. Vertical bars represent Mean ± SD.

Fig. 2. Serum copper concentrations (left side) and transferrin levels (right side) were higher in the BCAA group than in the control group (p < 0.05). Vertical bars represent Mean ± SD.

Based on the nodule occupancy rate, the severity of tumor nodules was classified into 4 categories: minimal, < 20%; mild, 20 to 40%; moderate, 40 to 60%; and severe, > 60%. The severity of the nodular findings was moderate and severe in each of two rats in the control group, but only minimal or mild in all rats in the BCAA group (Table 2).

4) Biochemical tests

There were no significant differences in albumin or liver function values between the two groups. Serum copper concentrations and transferrin levels were higher in the BCAA group than in the control group (p < 0.05) (Table 3, Fig. 2).

Table 3. Laboratory Findings in Control and BCAA Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>BCAA</th>
<th><em>p</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum copper</td>
<td>20 ± 5</td>
<td>25 ± 6</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Transferrin</td>
<td>300 ± 20</td>
<td>350 ± 25</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Values are for male rats. *p* < 0.05 was considered statistically significant.
Fig. 3. Analysis of serum amino acids shows that some rats had high valine, leucine, and isoleucine levels but low tyrosine levels in the BCAA group. Fischer's ratio was higher in the BCAA group than in the control group, but the difference was not significant. Vertical bars represent Mean ± SD.

Fig. 4. Histological findings in liver of LEC rats, the control group (left side) had extensive hepatocellular carcinoma nodule formation (arrows) compared to the BCAA group (right side). BCAA group showed rather mild necroinflammatory changes than that of the control group in non-tumor areas. (Masson-trichrome stain x 2)

Fig. 5. Histological findings in liver of LEC rats, staining degree for iron and copper by Berlin-blue and HBs Orcein were mild in the BCAA groups (right side) compared to the control group. (left side) (top: HBs Orcein stain x2, bottom: Berlin-blue stain x2)
Analysis of serum amino acids in the BCAA group showed that some rats had high valine, leucine, and isoleucine levels but low tyrosine levels. Fischer's ratio was higher in the BCAA group than in the control group, but the difference was not significant (Fig. 3). There was no significant correlation between the blood Fischer's ratio and the severity of tumor nodules.

5) Histological examination

Compared to the control group, more rats in the BCAA group had less extensive HCC nodule formation per unit area (Fig. 4). The findings were similar to those for HCC nodule formation on the liver surface. There were some rats in the BCAA group with mild necroinflammatory changes in non-tumor areas. Staining degree for iron and copper using HBs orcein was mild in all rats compared to the control group (Fig. 5).

6) Tissue amino acid analysis

There were no significant differences in tissue amino acid levels. Fischer's ratio was higher in the control group than in the BCAA group, but the difference was not significant (Fig. 6). There was also no significant correlation between the tissue Fischer's ratio and the severity of tumor nodules.

4. Discussion

Serum albumin levels are an important prognostic factor in hepatic cirrhosis, and patients with decompensated liver cirrhosis and hypoalbuminemia are often given oral BCAA-supplementation as nutritional support therapy.

Liver cirrhosis may be associated with an amino acid imbalance, including decreased BCAAs and increased aromatic amino acids (AAAs). This amino acid imbalance can be assessed using Fischer's ratio (BCAA/AAA ratio). More recently, the BCAA/tyrosine ratio (BTR), which correlates well with Fischer's ratio, has been used in clinical practice as a more economical and rapid way of evaluating this imbalance. Long-term oral supplementation with BCAAs has been investigated in patients with compensated cirrhosis, where protein synthesis is relatively well preserved. In patients with serum albumin values of 3.6 to 4.0 g/dl and a BTR ratio of 4.00, oral supplementation with BCAAs for 1 year helped to maintain serum albumin levels compared to the control group. In Japan, the most important prognostic factor in patients with...
cirrhosis of the liver is the development of hepatocellular carcinoma. However, the effects of long-term oral supplementation with BCAAs on liver carcinogenesis in compensated cirrhosis patients have remained unknown.

The inbred strain of LEC rats was established from a closed colony of Long-Evans rats. Acute hepatitis in LEC rats is a hereditary disorder associated with abnormal copper overload in the liver, which is similar to the pathogenesis of human Wilson’s disease. About one-half of the rats die due to fulminant hepatitis and those that survive suffer from chronic hepatitis for over a year and ultimately develop cholangiofibrosis and HCC. The natural history of liver disease in LEC rats closely resembles that of human chronic hepatic disease in that HCC follows persistent hepatitis. Therefore, LEC rats are suitable models for evaluating the effects of long-term oral supplementation with BCAAs for human hepatocarcinogenesis.

In the present study, we housed 20 LEC rats starting at 10 weeks of age. By 17 to 23 weeks of age, 8 of the rats (40%) developed fulminant hepatitis and died. Twelve of the rats (60%) survived to 6 months of age. During the supplementation experiment, body weight did not differ significantly between the two groups, but the BCAA group rats tended to weigh more, suggesting a possibility of beneficial effect on the maintenance of nutritional status. The serum albumin levels did not significantly differ between the BCAA group and control group. There was no effect on improvement of hypoalbuminemia after the onset of acute hepatitis in LEC rats and no marked effect on protein synthesis. Although Fischer's ratio did not differ significantly between the two groups, 4 of 6 rats in the control group had ratios of 2.2 in contrast to higher ratios of 2.3 in all rats in the BCAA group. This suggests that administration of BCAAs helps to improve amino acid imbalance. Tumor nodules were observed in all rats in the BCAA group and control group. However, rats in the BCAA group had less extensive nodule formation on the liver surface per unit area. Thus, long-term oral supplementation with BCAAs did not promote carcinogenesis, and indeed, may have had an inhibitory effect on carcinogenesis. Amino acids in hepatic tissues were also evaluated, but there were no fixed trends between the BCAA and control groups. Watanabe et al. reported that amino acid content in human tumor tissue was generally higher than in the corresponding nontumorous tissues, especially in the case of HCC. The methionine, tyrosine, and phenylalanine contents in HCC were much higher than in cirrhotic or normal liver. Our findings may be due to sampling a mixture of tumor and non-tumor tissue.

In LEC rats, administration of copper chelating agents completely prevents carcinogenesis, decreases hepatic tissue copper levels, and increases urinary copper excretion. This strongly suggests that copper accumulation in liver tissue plays a role in hepatic carcinogenesis. Copper chelating agents also inhibit the development of cholangiofibrosis in non-tumor tissue. In our study, serum copper concentrations were higher in the BCAA group, and staining for copper was milder in hepatic tissue of the BCAA group compared to the control group. There were also several rats in the BCAA group with mild necrotic and inflammatory changes in non-tumor tissue. Our results suggest that BCAAs promote the release of copper into blood and prevent copper deposition in liver tissue. In addition, there were no differences in serum iron levels between the BCAA group and the control group, but transferrin, which is a useful maker of serum iron levels, was higher in BCAA group. Staining for iron in hepatic tissue of the BCAA group, like copper staining, was mild. The above findings show that long-term oral supplementation with BCAAs may have a chelating effect in LEC rat liver.

BCAA-supplementation therapy has been used to alleviate hypoalbuminemia in patients with decompensated liver cirrhosis. However, the mechanisms of the specific stimulatory effects of BCAAs on protein production by hepatocytes remain to be elucidated. One of the BCAAs, leucine, was recently reported to activate phosphorylation of two components involved in protein synthesis-p70 S6 kinase and eukaliotic initiation facter 4E binding protein pathways mediated by mTOR (mammalian target of rapamycin; an intracellular target of amino acids). Furthermore, leucine stimulates hepatocyte growth factor
(HGF) in hepatic stellate cells and may promote hepatocyte protein synthesis. The c-met proto-oncogene receptor has a high affinity for HGF, and activation of c-met leads to tumor cell proliferation. Therefore, the possible effects of BCAAs, mediated by HGF production, on hepatic carcinogenesis remains a concern. Further studies are needed to clarify this issue.

5. Conclusion

Long-term oral supplementation with BCAAs in LEC rats stimulated the release of copper into blood and inhibited the deposition of copper into hepatic tissue. BCAAs did not cause carcinogenesis, tumor growth, or tumor progression in LEC rats.

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25
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LEC ラット肝発癌モデルにおける
分枝鎖アミノ酸製剤長期投与の影響

抄録

分枝鎖アミノ酸（BCAA）製剤は、非代償性肝硬変患者の栄養管理に広く用いられている。しかし、慢性肝疾患における長期に渡る BCAA 製剤の投与が、肝細胞癌の発生および増殖進展にどのような影響を与えるかは不明であり、肝自然発癌モデルである LEC （Long Evans Cinnamon）ラットを用い検討した。6 カ月齢まで生存した LEC ラット 12 匹を、BCAA 群（6 匹）、control 群（6 匹）に分け、おのの BCAA 含有食、control 食にて 12 ヶ月齢まで飼育し実験に用いた。血清アルブミン、肝機能検査両群に差は認めなかった。血中銅およびトランスフェリンは、BCAA 群が control 群に比し高値（p < 0.05）を示した。Fisher 比は、BCAA 群が control 群に比し高値を認めるも両群間に有意差は認めなかった。両群すべてのラットで肝表面に灰白色調の硬い肝細胞癌結節が認められ、その程度は、control 群に比し BCAA 群は軽度であった。組織所見においても、BCAA 群は、癌結節の出現程度は少ないラットが多く、非腫瘍部は壊死炎症像の軽度のラットが多く存在し、鉄および HBs Orcein を用いた銅の染色性は control 群に比し軽度であった。LEC ラットにおける BCAA の長期投与は、血中への銅の放出を促し、肝組織への銅の沈着に対し抑制的に作用したと考えられ、発癌および癌の増殖、進展への関与は認められなかった。