Abnormal Increase in the Expression Level of Proliferating Cell Nuclear Antigen (PCNA) in the Liver and Hepatic Injury in Rats with Dietary Cobalamin Deficiency

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Summary Dietary cobalamin (Cbl; vitamin B12) deficiency resulted in severe growth retardation in rats, and body weight in the Cbl-deficient rats at 20 wk of age was significantly lower compared with the age-matched Cbl-sufficient control rats. In contrast, liver weight, when normalized to body weight, was greater in the Cbl-deficient rats than in the controls (p<0.05). The expression level of proliferating cell nuclear antigen (PCNA), a marker for cell proliferation, in the liver was significantly enhanced in the deficient rats, suggesting that cell proliferation is abnormally activated in the liver under Cbl-deficient conditions. In addition, plasma alanine aminotransferase (ALT) activity, a marker for hepatic injury, was also significantly elevated in the deficient rats. When l-carnitine, which is used clinically for the treatment of Cbl-deficient patients with methylmalonic aciduria, was administered to the Cbl-deficient rats by intraperitoneal injection twice per day for 2 wk (each 0.5 mmol), the amount of methylmalonic acid excreted into the urine was significantly reduced, and the plasma ALT activity was lowered to a normal level. However, the PCNA expression in the liver was barely influenced by the treatment with carnitine. In contrast, when the deficient rats were fed an l-methionine-supplemented diet (4 g of l-methionine per kg of diet) for 2 wk, the increased expression of PCNA was normalized.

Key Words cobalamin-deficient rats, proliferating cell nuclear antigen, liver injury, methylmalonyl-CoA mutase, methionine synthase

There are two kinds of cobalamin (Cbl; vitamin B12)-dependent enzymes in mammals (1): One of the two enzymes is methionine synthase in which methylcobalamin (MeCbl) functions as a cofactor, and the other is L-methylmalonyl-CoA mutase that requires 5-deoxyadenosylcobalamin (AdoCbl). In mammals under Cbl-deficient conditions, methionine synthase and methylmalonyl-CoA mutase activities are greatly lowered due to the defect in their coenzymes, MeCbl and AdoCbl, and hematological and neurological abnormalities and growth retardation are induced (1–3).

Methionine synthase catalyzes the synthesis of methionine from homocysteine with 5-methyltetrahydrofolate as a methyl donor (1). In addition to protein synthesis, methionine, after being converted to S-adenosyl-methionine, is used as a methyl donor in various biological transmethylation reactions. Furthermore, methionine synthase is the sole enzyme that converts 5-methyltetrahydrofolate to tetrahydrofolate (4). Thus, folate and C1 metabolism are impaired in mammals with Cbl deficiency in which methionine synthase activity is lowered. Hematological abnormality observed in Cbl-deficient patients (that is, megaloblastic anemia) is thought to be due to the decrease in methionine synthase activity, because thymidylate and purine biosynthesis requires folate coenzyme (5).

L-Methylmalonyl-CoA mutase, which catalyzes the isomerization of L-methylmalonyl-CoA to succinyl-CoA, is the final enzyme in the propionate pathway in mammals, whereby propionyl-CoA, derived from the catabolism of branched-chain amino acids, odd-numbered fatty acids, thymine, uracil and cholesterol, is converted to succinyl-CoA for use in the citric acid cycle (1). Under Cbl-deficient conditions, due to the decrease in the enzyme activity, propionyl-CoA, methylmalonyl-CoA and methylmalonic acid are accumulated in the plasma and tissues, leading to the disruption of normal metabolism in mammalian cells (1, 2, 6). In particular, respiratory activity is decreased in the liver under Cbl-deficient conditions, because of the inhibition of succinate dehydrogenase by methylmalonic acid (2, 7). The decrease in respiratory activity in the liver is thought to

Abbreviations: AdoCbl, 5-deoxyadenosylcobalamin; ALT, alanine aminotransferase; Cbl, cobalamin; CN-Cbl, cyanocobalamin; MeCbl, methylcobalamin; PCNA, proliferating cell nuclear antigen.

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Infancy (acidosis that may result in brain damage and death in biological fluids causes severe and recurrent metabolic thermore, the accumulation of methylmalonic acid in causes severe growth retardation in mammals. Furthermore, the accumulation of methylmalonic acid in Cbl-deficient conditions compared with the Cbl-sufficient controls (11, 12). In addition, in sheep with Cbl deficiency induced by the depletion of cobalt in their diet, abnormal accumulation of lipids and functional disorder are observed in the liver (13–15). However, mechanisms by which these abnormalities observed in the liver are induced by Cbl deficiency have not yet been well studied.

In the present study, we have found that the expression of proliferating cell nuclear antigen (PCNA), a marker for cell proliferation (16, 17), in the liver is significantly elevated in rats with dietary Cbl deficiency, and we suggest that cell proliferation is abnormally activated in the liver as a result of the decrease in the methionine synthase activity under Cbl-deficient conditions. In addition, it is also reported that liver injury occurs in the Cbl-deficient rats, presumably through the toxic effects of propionyl-CoA, methylmalonyl-CoA and methylmalonic acid accumulated as a result of the decrease in methylmalonyl-CoA mutase activity.

MATERIALS AND METHODS

Animals and treatments. Male Wistar rats (3 wk old), born to 14-wk-old parent rats which had been fed a Cbl-deficient diet ad libitum for 8 wk, were used. The parent rats were obtained from Kiwa Laboratory Animals (Wakayama, Japan). The Cbl-deficient diet contained the following (per kg of diet): 400 g of defatted soybean (of which about 50% was crude protein and the remainder carbohydrates; obtained from Showa Sangyo, Tokyo, Japan), 4.35 g of glucose, 100 g of soybean oil, 35 g of a mineral mixture (AIN-93G-MX; CLEA Japan, Inc., Tokyo, Japan), 10 g of a Cbl-free vitamin mixture (AIN-93VX without Cbl; CLEA Japan, Inc.) and 2 g of choline chloride. All experimental procedures involving laboratory animals were approved by the Animal Care and Use Committee of Osaka Prefecture University.

The weanling rats at 3 wk of age were weighed, and based on their body weight data, they were randomly allocated to three groups: Cbl-deficient, Cbl-sufficient pair-fed control and Cbl-sufficient ad libitum control groups. The rats involved in the Cbl-deficient and Cbl-sufficient ad libitum control groups were allowed free access to the Cbl-deficient and -sufficient diets, respectively. The Cbl-sufficient diet was identical to the deficient one, except that cyanocobalamin (CN-Cbl) was included at 25 μg per kg of the diet. Food consumption by each rat in the Cbl-deficient group was recorded daily, and on the basis of this food consumption the rats in the Cbl-sufficient pair-fed control group were pair-fed with the Cbl-sufficient diet. These rats were housed individually at controlled temperature (22±2°C), humidity (55±10%) and lighting (from 08:00 to 20:00), and water was given freely. At 20 wk of age, blood was collected from inferior vena cava in these rats under diethyl ether anesthesia, and liver, kidneys, heart and spleen were obtained and weighed. To measure the amount of methylmalonic acid excreted into urine, the urine was sampled from these rats, which had been moved to individual metabolic cages at 19 wk of age, during the last 24 h of their life.

In a Cbl-supplementation experiment, the Cbl-deficient rats at 20 wk of age were fed a Cbl-supplemented diet (in which CN-Cbl was added at 100 μg per kg of the diet) for 2 wk. At the end of the feeding, urine was sampled from these rats during the last 24 h of their life in individual metabolic cages, blood was collected under diethyl ether anesthesia, and the liver was excised. For administration of carnitine into the Cbl-deficient rats, 0.5 mmol of L-carnitine (kindly donated by Hamari Chemicals, Osaka, Japan), which had been dissolved in 2 mL of water and adjusted to pH 7.5, was intraperitoneally injected twice per day for 2 wk. In addition, methionine was supplemented into the Cbl-deficient rats, by adding L-methionine at 4 g per kg to their diet for 2 wk.

Measurements of urinary methylmalonic acid concentration, hepatic triacylglycerol content, and plasma alanine aminotransferase (ALT) activity. Methylmalonic acid concentration in urine was determined by high-performance liquid chromatography according to a previous paper (18). Triacylglycerols in the liver were extracted with isopropanol and measured using a commercial kit (Type Wako TG-H; Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer’s instructions. Plasma ALT activity was determined using a commercial kit (Transaminase CII Test Wako; Wako Pure Chemical Industries, Ltd.).

Western blot analysis of PCNA. The liver, kidneys or heart (about 0.3 g) were homogenized with a Teflon homogenizer in 3 mL of 25 mM Hepes-NaOH buffer, pH 7.5, containing 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/mL leupeptin, 1 μg/mL aprotonin, 1 mM EDTA and 1% Nonidet P-40, at 4°C. After centrifugation at 15,000×g for 15 min, the supernatant obtained was used as a sample solution. The sample solution was subjected to SDS-polyacrylamide gel electrophoresis (15% gel), and proteins in the gel were transferred to a polyvinylidene difluoride membrane by electroblotting. The membrane was probed with an anti-PCNA monoclonal antibody (DAKO, Glostrup, Denmark), and immunoreactive proteins on the membrane were detected with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies by a chemiluminescent method using a commercial kit (Super Signal West Pico Chemiluminescent substrate system; Pierce). Digitized images were obtained with a luminescent image analyzer (LAS-1000 plus; Fuji Film, Tokyo, Japan), and band intensities were quantified.

Statistical analyses. Data were evaluated by one-way
Liver 2.82
Kidneys 0.483

body weight in the Cbl-deficient rats (387 as means±SD, and statistical significance is defined as p<0.05.

RESULTS AND DISCUSSION

Severe growth retardation occurred when weanling rats (3 wk old) were fed a Cbl-deficient diet for 17 wk, as has been observed previously (2, 18). At 20 wk of age, body weight in the Cbl-deficient rats (387±40 g) was significantly lower, even compared with that in rats involved in the Cbl-sufficient pair-fed control group (529±109 g). In contrast, as shown in Table 1, liver weight, when normalized to body weight, was greater in the Cbl-deficient group than in either the Cbl-sufficient pair-fed or ad libitum control group (p<0.05), suggesting that Cbl deficiency results in enlargement of the liver. Kidney weight per unit of body weight was also significantly greater in the Cbl-deficient group compared with the two Cbl-sufficient control groups, but significant difference due to Cbl deficiency was not observed in the heart or spleen weights when normalized to body weight. These results are almost consistent with previous reports (11, 12).

The level of PCNA expression was examined as a marker for cell proliferation (16, 17) in the liver, kidneys and heart by Western blotting. As shown in Fig. 1, Cbl deficiency resulted in a substantial increase in the PCNA expression in the liver. When the relative amount was estimated with an image analyzer, the level of hepatic PCNA expression in the Cbl-deficient rats at 20 wk of age was about 2 fold that of the Cbl-sufficient ad libitum control rats at the same age (p<0.05) (Fig. 2A). In contrast, the levels of PCNA in the kidneys and heart were barely affected by Cbl deficiency (Fig. 1). It is thus suggested that cell proliferation is abnormally activated in the liver (but not the kidneys or heart) when Cbl is depleted in rats. The abnormality in cell proliferation may relate to the enlargement of the liver observed in the Cbl-deficient rats; however, details remain obscure.

When the Cbl-deficient rats (20 wk old) were fed a Cbl-supplemented diet, in which CN-Cbl was included at 100 μg per kg of the diet, for 2 wk, the urinary excretion of methylmalonic acid, an index of Cbl deficiency, disappeared almost completely (Fig. 2C), as has been reported previously (18). In the Cbl-supplemented rats, the level of hepatic PCNA expression was decreased to near the level of the Cbl-sufficient rats (Fig. 2A).

It has been reported that functional disorder occurs in the liver, accompanying the abnormal accumulation of triacylglycerols and free fatty acids, when Cbl deficiency is induced in sheep by the depletion of dietary cobalt (13–15). To examine whether hepatic injury is induced by Cbl deficiency in rats as well as sheep, plasma ALT activity, a specific marker for hepatic injury, was compared between the Cbl-sufficient and -deficient rats. As shown in Fig. 2B, the plasma ALT activity was increased 3 fold in the Cbl-deficient rats, compared with

Table 1. Liver, kidney, heart and spleen weights in Cbl-deficient rats.

<table>
<thead>
<tr>
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<th>Ad libitum control</th>
<th>Pair-fed control</th>
<th>Cbl-deficient</th>
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<tr>
<td></td>
<td>(% of body weight)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>2.82±0.23ab</td>
<td>2.65±0.27b</td>
<td>3.81±0.27a</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.483±0.047ab</td>
<td>0.534±0.063b</td>
<td>0.807±0.104a</td>
</tr>
<tr>
<td>Heart</td>
<td>0.229±0.025ab</td>
<td>0.246±0.027b</td>
<td>0.282±0.026a</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.150±0.015ab</td>
<td>0.161±0.013a</td>
<td>0.175±0.026b</td>
</tr>
</tbody>
</table>

Liver, kidney, heart and spleen weights were determined in the Cbl-sufficient ad libitum control, Cbl-sufficient pair-fed control and Cbl-deficient rats at 20 wk of age, and data (mean±SD, n=7) are presented as % of body weight (body weights in these rats were 655±72, 529±109 and 387±40 g, respectively). Values with different superscript letters are significantly different (p<0.05).

Fig. 1. Western blot analyses of PCNA in the liver, kidneys and heart of the Cbl-deficient rats. The homogenates of the liver (10 μg of protein), kidneys (100 μg of protein) and heart (150 μg of protein), prepared from the Cbl-sufficient ad libitum control or Cbl-deficient rats (20 wk old), were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with an anti-PCNA monoclonal antibody.
the Cbl-sufficient ad libitum control rats (p<0.05). Furthermore, the increased ALT activity in the deficient rats was normalized by the supplementation of Cbl for 2 wk. The triacylglycerol content in the liver was also greater in the Cbl-deficient group (26.0±7.1 μmol/g liver) than in the Cbl-sufficient ad libitum control group (16.2±4.5 μmol/g liver) (p<0.05). However, the increase in the triacylglycerol content due to Cbl deficiency in rats (1.6-fold) was less pronounced compared with that reported in sheep (7-fold) (14). In addition, as shown in Fig. 3, no apparent difference was observed in the liver among the Cbl-sufficient ad libitum control, Cbl-deficient and Cbl-supplemented groups.

When methylmalonyl-CoA mutase activity is de-
increased due to the depletion of its coenzyme, AdoCbl. The hepatic levels of propionyl-CoA and methylmalonyl-CoA are greatly increased, resulting in the abnormalities in pyruvate and fatty acid oxidation, gluconeogenesis and ureagenesis in the liver (6, 19–21). In addition, methylmalonic acid is produced by the hydrolysis of methylmalonyl-CoA, accumulated in the plasma and tissues, and excreted abnormally into the urine (methylmalonic aciduria) (1, 2, 16). It has been reported that methylmalonic acid, when accumulated in the liver, decreases respiratory activity by inhibiting the succinate dehydrogenase reaction (2, 7). There is a possibility that the toxic effects of propionyl-CoA, methylmalonyl-CoA and methylmalonic acid are involved in mechanisms by which Cbl deficiency results in the abnormal activation of cell proliferation in the liver and in hepatic injury. To explore this possibility, L-carnitine (0.5 mmol) was administered to the Cbl-deficient rats by intraperitoneal injection twice per day for 2 wk. It has been reported that when a large amount of carnitine is administered to rats with Cbl deficiency, propionyl-CoA and methylmalonyl-CoA are converted to non-toxic propionyl-carnitine and methylmalonyl-carnitine, respectively, and excreted into urine (22); L-carnitine is clinically used for therapy in patients with methylmalonic aciduria (23). As shown in Fig. 4C, when the Cbl-deficient rats were treated with carnitine for 2 wk, the amount of methylmalonic acid in urine was reduced to about 35% of that before the treatment. In the Cbl-deficient rats treated with carnitine, plasma ALT activity was lowered to the level of the Cbl-sufficient rats (Fig. 4B). It is thus suggested that hepatic injury occurs in the Cbl-deficient rats as a consequence of the decrease in methylmalonyl-CoA mutase activity that results in the accumulation of toxic propionyl-CoA, methylmalonyl-CoA and methylmalonic acid. However, the level of hepatic PCNA expression was barely decreased by the administration of carnitine to the Cbl-deficient rats (Fig. 4A).

Methionine synthase, which catalyzes the synthesis of methionine from homocysteine, requires MeCbl as a cofactor, and its activity is lowered in mammals under Cbl-deficient conditions (1). To compensate for the decrease in methionine synthase, the Cbl-deficient rats were fed a methionine-supplemented diet (in which L-methionine was added at 4 g per kg of the diet) for 2 wk. As shown in Fig. 5, the increased expression of PCNA in the liver was normalized when methionine was supplied to the Cbl-deficient rats. Lu et al. (24) have reported that the expression of growth-related genes, including early growth response 1 and PCNA, is increased in the liver of methionine adenosyltransferase 1A knockout mice, in which S-adenosyl-methionine is decreased in the liver. The synthesis of S-adenosyl-methionine is thought to be affected under Cbl-deficient conditions in which methionine is not sufficiently provided from homocysteine due to the decrease in methionine synthase activity. Indeed, it has been observed that the concentration of S-adenosyl-methionine is lowered, with a concomitant increase in the level of S-adenosyl-homocysteine, in Cbl-deficient animal models or humans (1). It is thus reasonable to postulate that the expression of PCNA is abnormally increased in the liver as a result of the decrease in the level of S-adenosyl-methionine in the Cbl-deficient rats, although details remain to be elucidated.

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