Vitamin B12 (B12) is thought to be the animal protein factor per se or a closely related substance (1), and its daily requirement is the smallest of all vitamins. The biochemical role of B12 is to be a cofactor for several enzymes. In mammals, methylcobalamin and adenosylcobalamin work as cofactors for methionine synthase (MS) and methylmalonyl-CoA mutase, respectively. Insufficient intake of B12 results in dysfunction of these enzymes, which causes characteristic B12-deficient symptoms, such as megaloblastic anemia, homocysteinemia, and methylmalonic aciduria. To successfully incorporate the very limited dietary sources of B12 into its target enzymes requires specific binding proteins and receptors for absorption, transportation, and activation. Disruption of this system by dysfunction of proteins due to gene mutations can cause similar symptoms.

Recently, many researchers have turned their attention to epigenetics. Chemical modification of DNA and histone with methyl groups may dramatically alter gene expression, which can induce cellular differentiation. These methyl groups are donated from S-adenosylmethionine (AdoMet), active methionine. While methionine is one of the essential amino acids in mammals, it can be re-synthesized by methylation of homocysteine using a methyl group on methyltetrahydrofolate (CH3-H4folate).}

\[ \text{CH}_3\text{-H}_4\text{folate} + \text{homocysteine} \rightarrow \text{tetrahydrofolate} + \text{methionine} \] (1)

The reaction catalyzed by MS (Eq. (1)) is responsible for two pathways: production of tetrahydrofolate for a...
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is impaired by further feeding of a B12-deficient diet. Evidenced damage to spermatogenesis and sperm maturation.

dietary B12-deficiency during gestation induces irreversible activity. Recently, Watanabe et al. have suggested that would not complement deficiencies of this enzyme to succinyl-CoA. Thus methionine supplementation catalyzes the rearrangement of methyl-malonyl CoA.

severely B12-deficient animal model by feeding rodents a soy protein-based B12-deficient diet (18% soy protein).

epigenetics may be critical for rational understanding.

To examine the action of B12, we have established a severely B12-deficient animal model by feeding rodents a soy protein-based B12-deficient diet (5–8). The B12-deficient rats and mice show remarkable damage to the testis (9–12), which can be almost completely prevented by supplementation of methionine to the diet (1,3).

Although the biochemical bases of several B12-deficiency symptoms have been poorly understood, considering the effect of these metabolic malfunctions on epigenetics may be critical for rational understanding.

To examine the action of B12, we have established a severely B12-deficient animal model by feeding rodents a soy protein-based B12-deficient diet (5–8). The B12-deficient rats and mice show remarkable damage to the testis (9–12), which can be almost completely prevented by supplementation of methionine to the diet (1,3). These findings suggest that the tissue injury is caused by dysfunction of MS, rather than methylmalonyl-CoA mutase. Methylmalonyl-CoA mutase is the other B12-dependent enzyme in mammals, and this enzyme catalyzes the rearrangement of methyl-malonyl-CoA to succinyl-CoA. Thus methionine supplementation would not complement deficiencies of this enzyme activity. Recently, Watanabe et al. have suggested that dietary B12-deficiency during gestation induces irreversible damage to spermatogenesis and sperm maturation is impaired by further feeding of a B12-deficient diet after weaning (14, 15). The hypothesis that impaired testicular development could be rescued by a methionine-enriched diet has not yet been examined. Here, we report effects of maternal intake of B12 and methionine on testicular morphology of offspring.

Materials and Methods

Animals and diet. As parent rats, 10-wk-old Wistar strain male and female rats (CLEA Japan, Inc., Tokyo, Japan) were purchased. Rats were bred with free access to food and water under constant temperature (22±3°C) and a 12-h cycle of light and dark. All protocols complied with the Guideline for Animal Experimentation (16). A B12-deficient diet was prepared according to our previous reports (5, 7, 8, 13). Briefly, diet composition (per kg) was as follows: soy protein (Ajinomoto Co., Ltd.), 100 g; a mineral mixture (8, 13), 35 g; a water-soluble vitamin mixture without B12 (8, 13), 10 g; choline chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 1.5 g. A methionine-enriched B12-deficient diet contained 0.5% dl-methionine (Wako Pure Chemical Industries, Ltd.), which was prepared by adding 5 g dl-methionine per 1 kg of the B12-deficient diet. Total amount of the diet was balanced by reducing the amount of anhydrous glucose to 668.5 g (8, 13). Control rats were fed a control diet, which contained cyanocobalamin (CN-B12, Wako Pure Chemical Industries, Ltd., 1 µg CN-B12/kg diet) in the B12-deficient diet.

Experimental design. The experimental schedule is summarized in Fig. 1. After copulation, maternal rats were divided into three groups: −B12, +B12 and +B12. Offspring born to dams fed the B12-deficient diet and the methionine-enriched B12-deficient diet were named −B12 and −B12+Met, respectively. As a control group, maternal rats who were fed the B12-deficient diet but who received CN-B12, were designated as +B12. They were individually housed and fed the same diets throughout gestation and lactation for 45 d. Male offspring were used for testicular morphology at the end of lactation and their body weight was measured at that time. Testes were fixed in neutralized formalin and then embedded in paraffin. Sections at 4 µm thickness were cut and stained with hematoxylin and eosin.

Statistical analysis. One-way ANOVA and Kruskal-Wallis tests using Stat View (version 5.0; SAS Institute.
Cary, NC) or Excel Tokei (Version 5.0; Esumi Co., Tokyo, Japan) were used for data analysis. Scheffe and Steel-Dwass tests were employed for evaluation of differences among groups. Differences were considered significant when p values were less than 0.05. Data are expressed as mean ±SD.

**Results**

Testicular weight of offspring born to dams fed the B12-deficient diet with (−B12+Met) or without methionine (−B12) at the end of lactation is shown in Table 1. Although the testicular weight of offspring in the −B12 group was lighter than that in the other two groups, there is no difference in three groups when they were compared with ratios of testicular weight per 100 g body weight. This was because offspring in the −B12 group did not grow as well as those in the two other groups.

Testicular morphology in the −B12 and −B12+Met groups is shown in Fig. 2. In the −B12 group (Fig. 2A and B), approximately 25% of the seminiferous tubules lacked healthy spermatocytes. The number of spermatoocytes was greatly reduced, although some pachytene spermatocytes, which can be found in the very early stage of meiosis, were observed. The ratio of apoptotic cells (likely derived from spermatogonia and/or sertoli cells) to all germ cells was very high. In contrast, the ratio was greatly reduced in the −B12+Met group (Fig. 2C and D). Moreover, the diameters of seminiferous tubules in the −B12+Met group were seemingly larger than those in the −B12 group. Indeed, the testicular morphology of the −B12+Met rats was more similar to that of normal rats (Fig. 2E). These observations indicate that methionine supplementation to the diet prevented the testicular damage, although full remediation was not achieved.

**Discussion**

Clarifying the contributions of folate and methionine metabolism to human health is quite difficult because various environmental conditions including nutritional and genetic diversities could affect this metabolism and its regulation. Folate fortification is likely to lower the concentrations of homocysteine in blood. However, why elevated levels of homocysteine are associated with cardiovascular disease and how adequate folate prevents neural tube defects are poorly understood. Here, we demonstrated for the first time roles of B12 and methionine on maternal nutrition using classic nutritionally deficient rats as a model. In this and our previous (13) studies, we showed the strong remedial effect of methionine on B12-deficiency, indicating that the function of MS in the testis would be important for tissue development. In fact, activity of the enzyme in the testis is higher than those in most of the other organs (13). Since B12 is the essential cofactor for MS and the diets contained sufficient folate, we focus on maternal nutrition of B12 and methionine in the following discussion.

The biological half-life of B12 is approximately 1 y (17–19), and this is the reason why raising animals in B12-deficiency is difficult, especially in a short period. We have learned that it takes more than 90 d to induce B12-deficiency in mature rats by feeding the soy protein-based B12-deficient diet after weaning (5, 10). However we found that the offspring of rats fed a B12-deficient diet for only the 45 d of gestation and lactation showed significant damage to the testes in this experiment, suggesting an increased requirement of B12 during gestation.
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In mature male rats born to dams fed the B12-deficient diet, found impaired sperm quantity and quality is irreversible in the testis of the infant, this could yield for further development of the tissue. If the damage were to be inaccurate. However, it can be presumed that higher content of B12 in rat milk during lactation is needed for neonates to undergo massive gains in body weight and full development of the testis in a short period.

Under the conditions of our experiments, reserve pools of B12 in the dam could be used to supply the fetus in utero, even when the dam is on a B12-deficient diet. For instance, in humans, blood B12 concentration in the fetus is ~3 times higher than that in the mother (22). After delivery, nursing is the only way to provide nutrients from dam to neonate. It would be difficult to provide such a high concentration of B12 in milk (see above) without adequate intake of B12. Both exhaustion of B12 and subsequently resulting low-production of methionine due to dysfunction of MS in the dam would result in the failure of testicular development of the neonate. Methionine supplementation could be beneficial for increasing free methionine and producing milk protein, consequently improving protein nutrition of dam and neonate. Similar to the testicular damage of the mature rat on a B12-deficient diet, disrupted transmethylation would explain the neonatal testicular damage, as discussed in our previous paper (13).

We have previously reported that damaged testicular morphology in B12-deficient mature rats cannot be completely recovered by administration of CN-B12 for 30 d (11). Similarly, damage to spermatogonia due to B12-deficiency in a pre-weaning period could be critical for further development of the tissue. If the damage were reversible in the testis of the infant, this could yield individuals infertile for the rest of their lives. Watanabe et al. (14) found impaired sperm quantity and quality in mature male rats born to dams fed the B12-deficient diet from gestation, although no apparent damage in testicular histology was found in the neonate at 0 d postpartum (15). As we mentioned above, B12 functions in quite small amounts and has a long biological half-life. The fetus could drain B12 from dam during gestation (see above), implying that it will be quite difficult to raise a fetus in B12-deficiency within 22 d (the gestation period). Thus, it is likely that the testicular injury of offspring chiefly resulted from methionine deficiency in the lactation period, which was caused by dysfunction of MS due to B12-deficiency. In fact, the Mtr (gene name for MS)-deficiency (Mtr−/−) mouse is an embryonic lethal (23), indicating absolute requirement of MS in the gestation period for fetal survival.

Offspring born to the B12-deficient dam were grown under a condition expected to lead to suppressed transmethylation, which might lead to hypomethylation of DNA, protein, and other methyl group acceptors in every organ. Extremely low levels of methionine would be difficult to achieve even if methionine (or methyl donor)-derived diets were prepared, because the pathway of methionine re-synthesis by methylenetetrahydrofolate reductase and MS would be activated under these conditions. Therefore, offspring in this study could be a novel model to investigate effects of a lowered-level of methionine in the pre-weaning period on development of infants, although biochemical parameters have to be determined. In our routine protocol, we use a casein-based B12-deficient diet instead of the soy protein diet during gestation and lactation (5). The casein diet contains a comparable amount of methionine to the methionine-enriched soy protein diet (13), suggesting that testicular damage before weaning would be minimized. Thus, the post-weaning young B12-deficient rat raised by our method would be a suitable model to investigate the biochemical basis of infertility caused by B12-deficiency.

Acknowledgments

KY thanks Dr. Rowena Matthews, the University of Michigan, for her helpful comments during the preparation of this manuscript.

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