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Review

Effects of Vitamin B_{12} and Folate Deficiencies on DNA Methylation and Carcinogenesis in Rat Liver

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Deficiencies of the major dietary sources of methyl groups, methionine and choline, lead to the formation of liver cancer in rodents. The most widely investigated hypothesis has been that dietary methyl insufficiency results in abnormal DNA methylation. Vitamin B_{12} and folate also play important roles in DNA methylation since these two coenzymes are required for the synthesis of methionine and S-adenosyl methionine, the common methyl donor required for the maintenance of methylation patterns in DNA. The aim of this study was to review the effects of methyl-deficient diets on DNA methylation and liver carcinogenesis in rats, and to evaluate the role of vitamin B_{12} status in defining carcinogenicity of a methyl-deficient diet. Several studies have shown that a methyl-deficient diet influences global DNA methylation. Evidence from *in vivo* studies has not clearly established a link between vitamin B_{12} and DNA methylation. We reported that vitamin B_{12} and low methionine synthase activity were the two determinants of DNA hypomethylation. Choline- or choline/methionine-deficient diets have been shown to cause hepatocellular carcinoma in 20–50% of animals after 12–24 months. In contrast, the effect of vitamin B_{12} withdrawal, in addition to choline, methionine and folate, induced hepatocellular carcinoma in less than 5% of rats. Clin Chem Lab Med 2003; 41(8):1012–1019

Key words: Cancer; Cobalamin; Gastrectomy; Liver; Methyl-deficiency; Rat.

Abbreviations: MTHFR, 5,10-methylenetetrahydrofolate reductase (EC 1.5.1.20); MTR, methionine synthase.

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Introduction

Dietary choline and lipotrope deficiencies are among the most studied pathological processes induced by nutritional modifications in experimental animals (1). Since 1946, the long-term administration of diets deficient in choline has been known to cause liver cancer in rats (2). Even though such diets were later found to be contaminated with aflatoxin, the role of a methyl-deficient diet in liver carcinogenesis has since then been evaluated (3–6). It is now well established that deficiencies of the major dietary sources of methyl donors, methionine and choline, lead to the formation of liver cancer in rodents (7). Several plausible mechanisms have been proposed to explain the enhancing effect of methyl deprivation on carcinogenesis (4, 6). The most widely investigated hypothesis was that dietary methyl insufficiency results in abnormal DNA methylation subsequent to the development of cellular methyl insufficiency (7). Different studies have used diets deficient in choline and methionine with or without vitamin B_{12} (using a deficient diet or total gastrectomy) or folate (deficient diet), in the presence or absence of carcinogens (8–13) or drugs impairing the 5,10-methylenetetrahydrofolate metabolism (one carbon metabolism) (14, 15) (Figure 1).

The use of carcinogens and/or specific drugs has been considered as controversial since these substances could be responsible for liver tumour emergence by direct effects and not through abnormal DNA methylation. Thus, we will review in this study the role of methionine metabolism on DNA methylation and the effects of global DNA methylation on carcinogenesis. Lastly, we will focus our Review on rat models assessing i) the effects of the use of carcinogens on liver carcinogenesis, ii) the role of diets deficient in choline and methionine with or without vitamin B_{12}/folate on liver carcinogenesis, and iii) effects of total gastrectomy on vitamin B_{12} levels.

Methionine Metabolism and DNA Methylation

Vitamin B_{12} and folate play important roles in DNA methylation (16). These two coenzymes are required for the synthesis of methionine and S-adenosyl methionine, the common methyl donor required for the maintenance of methylation patterns in DNA (17). Methylation of the 5-carbon on the cytosine residue is catalyzed by DNA methyltransferase enzymes, which use a methyl group from S-adenyl methionine. S-adenyl methionine derives from methionine, which

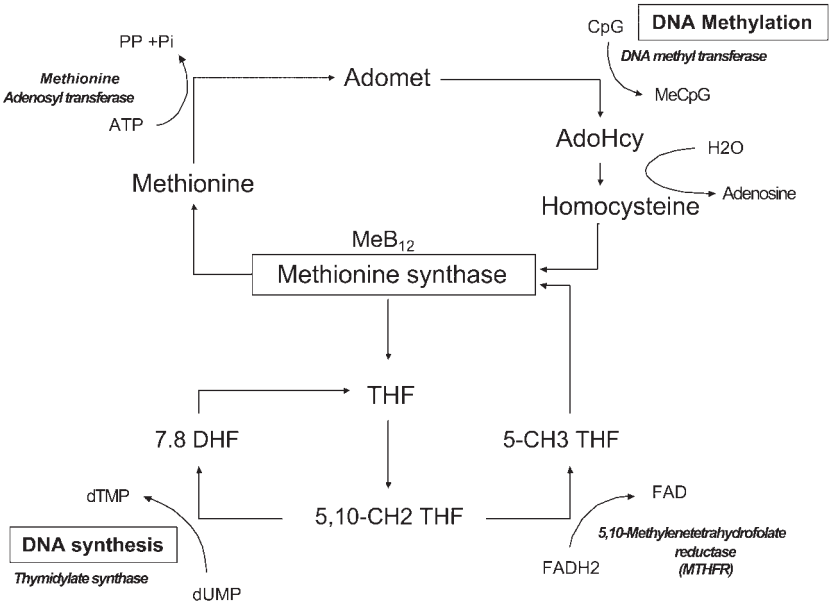

Figure 1 Diagram showing that methionine is synthesized by transmethylation of homocysteine by methionine synthase in the presence of vitaminic coenzymes, methyltetrahydrofolate and methylcobalamin. AdoHcy, adenosylhomocysteine; Adomet, adenosylmethionine; ATP, adenosine triphosphate; FAD, flavine adenine dinucleotide; Pi, phosphate; PP, pyrophosphate; THF, tetrahydrofolate.

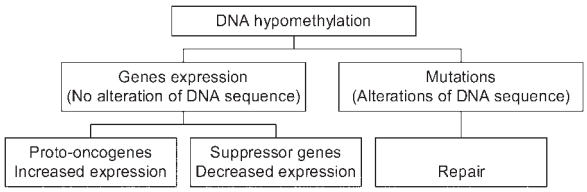

Figure 2 Relationship between DNA methylation and epigenetic control of gene expression and maintenance of genomic integrity.

is synthesized by methylation of homocysteine by methionine synthase (MTR) in the presence of coenzymes, methyltetrahydrofolate and methylcobalamin (18) (Figure 1). In the "normal" situation, 60% to 90% of all CpG sequences in the genome are methylated, whereas unmethylated CpG dinucleotides are mainly clustered in CpG-rich sequences, termed "CpG islands", and highly represented in gene promoter regions or initial exons of genes (19). DNA methylation is a fundamental mechanism for the epigenetic control of gene expression and the maintenance of genomic integrity (Figure 2). Therefore, an evaluation of genomic DNA methylation status is important for the study of cell growth regulation, tissue-specific differentiation and carcinogenesis (19).

Several studies suggest that nutritional status influences global DNA hypomethylation. However, the influence of nutrition on gene-specific DNA methylation is less clearly understood (20). Dietary factors that are involved in one-carbon metabolism that are likely to have an impact on DNA methylation processes mainly include vitamin B_{12}, folate, methionine, and choline (21). When the concentration of vitamin B_{12} and methionine is low, S-adenosyl methionine synthesis is reduced, and methylation of DNA is theoretically reduced. However, evidence from *in vitro* cultures and *in vivo* studies has not clearly established a link between vitamin B_{12} deficiency, increased genomic instability, and DNA methylation (16). Poirier *et al.* reported that S-adenosylmethionine in normal subjects was negatively correlated with folate and vitamin B_6 levels, but vitamin B_{12} levels were not assessed in this study (7). In 64 healthy men aged between 50 and 70 years, the micronucleus index (chromosome early damage biomarker) was not significantly correlated with folate levels but there was a significant ($p = 0.013$) negative correlation with serum vitamin B_{12} (22). However, the level of unmethylated CpG was not significantly related to vitamin B_{12} status. We recently reported that total MTR activity (holo- + apoenzyme) was lowered with vitamin B_{12} levels below 200 pmol/l in F344 rats, and that vitamin B_{12} was the single independent determinant of low MTR activity by logistic regression analysis (23). Furthermore, we found that low MTR activity and low vitamin B_{12} (lower quartile) were the two determinants of DNA hypomethylation. Conclusive further experiments to confirm this issue have yet to be performed.

The major sources of methyl groups in foods come from methionine, from the one-carbon metabolism *via* methylfolate, and from choline (24). It is well established that the metabolic pathways of methionine, choline and folate are interdependent. For example, when exogenous methionine and choline are limited in the diet, more folate is needed for the remethylation of homocysteine to form methionine (6). Choline, methionine and folate metabolism interact at the point that homocysteine is converted to methionine (25). This tight interrelationship between these three dietary sources of methyl groups makes it important that all

three be assessed when studying diet and DNA methylation. Tetrahydrofolate deficiency induced by a dietary folate-free diet results in diminished hepatic total choline and decreased S-adenosylmethionine concentrations (24). It has been shown that animals fed diets deficient in choline and methionine have hypomethylated DNA (24, 26). It seems to be that all organs may have changes in global DNA methylation elicited by the nutritional status. However, most studies have assessed DNA methylation in the liver, gut, brain, kidney, leucocytes, and not elsewhere. Moreover, results from studies in rodents suggest that severe folate deficiency causes DNA hypomethylation but also DNA strand breaks with increased uracil and apurinic sites in DNA (16, 27, 28). Similar data have been reported in humans. A folic acid depletion study of nine postmenopausal women in a metabolic unit showed an increased DNA hypomethylation in lymphocytes (29). Cervical and gastric/colonic/rectal epithelium DNA methylation was also significantly correlated to serum and tissue folate concentration, respectively (16).

DNA Methylation and Carcinogenesis

Several recent human studies have linked deficiencies in vitamin B_{12}, folic acid, and methionine with increased risk of cancer in various organs. These include the colon, the breast, the cervix, the lung, the stomach, the pancreas, and multiple myeloma (7, 8, 10, 30, 31). The question thus arises as to whether such deficiencies exert their activities through diminished availability of S-adenosylmethionine and subsequent abnormal DNA methylation. The fundamental properties required to generate the characteristic malignant attributes associated with cancer cells are the ability to replicate without limitation, indifference to positive growth signals, disregard for growth inhibitory factors, evasion of programmed cell death, sustained angiogenesis, and the ability to invade and metastasize. Each of these traits is influenced by a gene or set of genes. Failure to express the gene correctly and produce functional regulatory proteins leads to the uncontrolled pattern of cell behaviour observed in a typical neoplasm. Much of the focus of molecular biological research has concentrated on investigating the role of genetic changes (direct alterations of DNA base sequence). Alternative mechanisms of gene modulation (DNA methylation) have been coming under scrutiny that, without disrupting the actual sequence of a gene, affect its expression and remain preserved after cell division (32). The most likely mechanisms through which global DNA hypomethylation may induce neoplastic transformation include activation of oncogenes, inactivation of tumour-suppressor genes, induction of genomic instability and increased mutability that result in abnormal chromosomal structures (16, 33). Thus, changes in methylation status can be considered as being a preestablished state that facilitates tumour initiation and/or progression.

DNA methylation is a heritable epigenetic event that is mediated by transfer of methyl groups. Despite the frequently observed cancer-associated increases of regional hypermethylation, the near universality of global DNA hypomethylation in many types of human cancer suggests that such hypomethylation plays a significant and fundamental role in tumourigenesis (20). Two patterns of altered methylation have been observed: wide areas of global hypomethylation along the genome, and localized areas of hypermethylation at certain specific sites, the CpG islands, within the gene promoter regions (34). Thus, in theory, decreased methylation, and hence relief of transcriptional silencing, may allow the expression of previously quiescent proto-oncogenes to become active and induce the cell proliferation events. Alternatively, increased methylation at previously unmethylated sites, such as the promoter regions of a tumour suppressor gene, may result in their silencing through inhibition of transcription and their inability to suppress cell proliferation (4, 20, 32, 35). Some observations from several groups have shown that the total 5-methylcytosine content of DNA is decreased during hepatocarcinogenesis and colonic carcinogenesis (4, 26, 32, 36). The hypomethylation of DNA also coincided with changes in mRNA levels of several genes such as *c-myc*, *c-fos*, *c-Ha-ras*, *c-Ki-ras*, and *p53* (3, 6, 11, 13, 36). In the same way, MTR and methyltetrahydrofolate reductase gene polymorphisms have been related to genomic hypomethylation and carcinogenesis (37–39). For example, carriers of the 5,10-methylenetetrahydrofolate reductase (EC 1.5.1.20) (MTHFR) C677T variant have been considered as being at increased risk of developing colorectal neoplasia (40).

Moreover, when methylation of DNA is reduced, inhibition by S-adenosylmethionine of methylenetetrahydrofolate reductase is minimised resulting in the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, thus favouring an increase in the dUMP pool and uracil incorporation into DNA (6, 16, 41). This dUMP accumulates and as a result uracil is incorporated into DNA instead of thymine. There is good evidence that this mechanism not only leads to point mutations but may also result in the generation of single- and double-stranded DNA breaks, chromosome breakage and micronucleus formation (42). Thus, abnormal methylation patterns can also indirectly affect gene activity with the disruption of the transcription-translation process by increasing the probability for a mutational event to take place and reducing overall chromosomal stability, resulting in the manufacture of a dysfunctional protein product (32). Several studies showed that the uracil level in DNA was 70-fold higher in individuals with serum folate <4 ng/ml relative to individuals with serum folate >4 ng/ml (16, 42). Other studies have evidenced DNA damage by measuring increased amounts of DNA-strand breaks in spleen cells of rats fed a methyl/folate-deficient diet (41).

Lastly, DNA methylation is also considered to have an effect on the cytosine deamination rate. In purified DNA, methylation of cytosines is known to increase

their rate of deamination by a factor 2 to 4. Therefore, it seems likely that one of the causes of mutational hot spots may simply be the increased generation of mismatches when the target cytosines are methylated (43).

Rat Model and Hepatocarcinoma

Use of diets with carcinogens

In the absence of carcinogen, the chronic feeding of the amino acid-defined diet, lacking both methionine and choline, led to the formation of altered hepatic foci that continuously increased in volume but not in number. This observation was consistent with the hypothesis that methyl deprivation is not a continuously initiating stress, but rather is a highly effective promoter of cancer occurrence in pre-existing initiated hepatocytes (4, 44). Other investigations have consequently been conducted on the interactions between hepatocarcinogens and liver tumour promoters and labile methyl groups. Two categories of chemical agents provide evidence that chemical carcinogenesis occurs in part through abnormal methylation processes: i) classical carcinogens and tumour promoters and ii) the anti-metabolites of methylation reactions (3). The first includes: 2-acetylaminofluore, diethylnitrosamine, phenobarbital, dichlorodiphenyltrichloroethane, nickel, arsenic, zinc deficiency, and methapyrilene; each of these agents alters either S-adenosylmethionine or DNA methylation levels (3, 4). The tumourigenic activities of 2-acetylaminofluore, diethylnitrosamine, phenobarbital, and dichlorodiphenyltri-chloroethane have been shown to be enhanced by dietary methyl group deficiency or inhibited by supplemental methyl donors (4, 9). All of the hepatocarcinogenic stressors whose activity was inhibited by methyl donors were also shown to decrease the hepatic content of S-adenosylmethionine in the livers of chronically fed rats. The anti-metabolites of methylation, ethionine and azacytidine, provide additional evidence for a causative role of abnormal methylation in carcinogenesis (3).

Finally, it is considered that dietary methyl-deficiency lowers the apparent threshold for chemical carcinogenesis in the liver (8, 9). Whereas severe deficiency induces marked fatty liver and cirrhosis, the enhancement of carcinogenesis requires only moderate deficiency that may not induce cirrhosis. Hepatocarcinogenesis may even be reduced by severe deficiency (45). Methyl-deficiency accelerates and magnifies the appearance of preneoplastic molecular and morphological changes in the target cells and, ultimately, accelerates the development of tumours (31). However, the use of carcinogens has been considered as controversial for studying liver carcinogenesis since it is impossible to differentiate the respective effects of these substances and of the restrictive diets in liver tumourigenesis *in vivo*.

Diets without carcinogens

Effects of diets deficient in choline and methionine (without carcinogens)

Male rats fed a diet deficient in choline and low in methionine develop hepatocellular carcinomas in the absence of carcinogens (8, 30). The diet most frequently used for long-term studies with the methyl-deficient rat model is the Lombardi diet, which is low in methionine and choline deficient but with adequate amounts of vitamin B_{12} and folate (with 9% peanut meal, 8% soy protein isolate and 1% casein) (46). Feeding rats this diet has been shown to cause hepatocellular carcinomas in 20–50% of the animals after 12–24 months (Table 1) (6, 30, 47).

While there is a strong indication that methionine/choline-deficient diets can serve as effective promoting agents in multistage hepatocarcinogenesis, it is controversial as to whether such diets may also cause initiation of individual hepatocytes (3, 6, 44). Another diet used for this methyl-deficient cancer model is an amino acid defined diet (only pure L-amino acids) without choline and low in methionine but with adequate amounts of vitamin B_{12} and folate. The rationale for this

Table 1 Studies reporting liver carcinogenic effects of methyl-deficient diets without the use of carcinogens in rats.

Authors	Year	Diet composition					n	Hepatocellular carcinoma	Delay (months)
		Choline	Methionine	Folate	B_{12}	Amino acid			
Nakae (47)	1992	–	+	+	+	nd	10	20%	12
Lombardi (46)	1988	–	+	+	+	nd	19	26%	16
Lombardi (46)	1988	–	+	+	+	nd	15	73%	12
Yokoyama (10)	1985	–	+	+	+	nd	37	22%	11
Nakae (47)	1992	–	+	+	+	AA-defined	10	100%	12
Mikol (8)	1983	–	–	+	+	nd	30	40%	19
Ghoshal (30)	1984	–	–	+	+	nd	45	51%	13–24
Henning (6)	1996	–	Low*	–	+	nd	10	100%	15
Hoover (9)	1984	–	–	–	–	nd	29	3.4%	12
Mikol (8)	1983	–	–	–	–	nd	30	0%	19

AA defined, choline-deficient L-amino acid defined diet; nd, not defined; * low methionine (0.23%).

modification is the unavoidable and unpredictable contamination of target nutrients by the protein sources of semi-purified diets (48). In comparison with the Lombardi diet, Nakae *et al.* showed that 100% of the rats fed the amino acid defined diet for 52 weeks developed hepatocellular carcinomas (30, 47). Their interpretation of the different effects on carcinogenesis of the two diets was that the diet providing whole proteins requires digestion. Peptides formed during digestion stimulate amino acid absorption and thus increase the methionine intake (6, 48). The tumourigenic activities of the amino acid-defined, methionine and choline-deficient diets were found in rats to be proportional to the corresponding decreases in the hepatic S-adenosylmethionine/S-adenosylhomocysteine ratios produced by the diets (49). These deficient diets also generate the presence of a new form of DNA methyltransferase (Dnmt; EC 2.1.1.73) activity in the liver tumours of rats fed the methionine and choline deficient diet, which could possibly explain in part the alterations in DNA methylation produced by the diet (50). Beside alterations of DNA methylation, genetic susceptibility and/or hormonal status are also important factors in liver tumourigenesis in rodents (3).

Interestingly, the hepatocarcinogenic activity of diets that were solely deficient in choline (diets adequate in methionine and folate content) has also been reported (1, 3). Deficiency alone is sufficient to trigger carcinogenesis with no need for exposure to any known carcinogen (25). It has been suggested that in addition to hypomethylation of DNA, the accumulation of 1,2-diacylglycerol and subsequent activation of protein kinase C within liver during choline deficiency is a critical abnormality that eventually contributes to the development of liver cancers (25).

Effects of diets deficient in choline, methionine and folate (without carcinogens)

Folate deficiency alone has little effect on hepatic lipid and carcinogenesis (31). A choline/methionine deficiency alone is able to generate a relative folate metabolic deficiency. Theoretically a combined folate-, choline- and methionine-deficient diet would have a stronger carcinogenic effect, compared to a diet only deficient in choline and methionine. However, there are only a few studies supporting this view (6). Henning *et al.* developed a folate/methyl-deficient diet, which also led to the typical symptoms of a choline deficient diet, such as fatty liver, decreased hepatic S-adenosylmethionine concentration and increased hepatic S-adenosylhomocysteine levels. This diet was based on 6% casein, 6% gelatine and 15% soy oil. It contained 0.23% methionine, 10 µg vitamin B_{12}/kg diet and no added choline or folate. Under this diet, 100% of rats developed hepatocellular carcinomas in a 12-month period (Table 1). They concluded that this diet was more severely methyl-deficient as compared to the Lombardi diet (6). Removal of folate, in addition to a choline-methionine-deficient diet, markedly increased the hepatic lipid content (51). Feeding a folate-choline-deficient and methionine-low diet also resulted in a similar decrease in the S-adenosylmethionine:S-adenosylhomocysteine ratio in the liver, compared to a diet deficient only in choline and methionine (52).

Effects of diets deficient in choline, methionine, folate and vitamin B_{12} (without carcinogens)

Kennedy *et al.* showed that dietary deficiency of vitamin B_{12} alone resulted in fatty liver in sheep (53). The effect of vitamin B_{12} withdrawal in addition to choline, methionine and folate in deficient diets has also been sparsely evaluated. In livers of rats fed a severely methyl-deficient diet (deficient in choline, methionine, folate, and vitamin B_{12}), decreased overall levels of DNA methylation were accompanied by simultaneous alterations in gene expression, yielding patterns that closely resembled those reported to occur in livers of animals exposed to cancer-promoting chemicals and in hepatomas (13). However, Poirier and co-workers showed that Fischer rats fed with this diet for 52 weeks without carcinogens had a hepatocarcinoma incidence of only 3.4% (9). We also confirmed this low occurrence since we found no liver tumour occurrence in a group of seven rats fed the same methyl-deficient diet for a median period of 20 months. All these rats underwent a liver magnetic resonance imaging at one year with a negative result. Animal livers harvested at death were free of tumour at pathology examination. However, a histological activity index was assessed in rat livers using a modified semi-quantitative score deriving from the Metavir and Working party of the World Congress of Gastroenterology scores (54, 55). Compared to controls (methyl adequate diet), these methyl-deficient diet rats had no significant difference of liver dysplasia score ($p = 0.39$). These results suggest that a severe methyl-deficient diet (deficient in choline, methionine, folate, and vitamin B_{12}) is probably not sufficient to promote tumour occurrence (Table 2). These results are in contrast with the effects of diets deficient in choline, methionine, and folate (without carcinogens) since this last diet is able to trigger liver tumours in more than 40% of rats (Table 1). However, this discrepancy between effects of choline-devoid and severe methyl-deficient diets (deficient in choline, methionine, folate, and vitamin B_{12} or lipotrope-deficient diet) was already noticed by some authors (1). They considered that choline-devoid diet induced frequent hepatocellular carcinomas ("50% to 70% of male rats by 2 years") and that the role of a completely methyl-deficient diet (deficient in choline, methionine, folate, and vitamin B_{12}) on liver carcinogenesis was uncertain. Thus, we hypothesized that vitamin B_{12} status was one of the crucial parameters that define carcinogenicity of a methyl-deficient diet in rats.

Surgical model (total gastrectomy)

We have developed a surgical model in rats to assess the role of severe vitamin B_{12} deficiency combined with a methyl-deficient diet on DNA methylation and liver carcinogenesis. We confirmed that vitamin B_{12} plasma

Table 2 Liver pathology and dysplasia score in rats that underwent a methyl-deficient diet (choline, methionine, folate) and/or B_{12} deficiency (total gastrectomy or B_{12}-deficient diet) (personal data).

Diet deficient in	Total gastrectomy	B_{12} deficiency	Median survival (quartiles) (months)	Liver MRI (at 1 year)	Mean liver dysplasia score (0–3)	Liver tumour pathology
Choline, methionine, folate	Yes	Severe	1.6 (1.4–3.4)	–	0 (±0)	No tumour
No deficiency	Yes	Severe	2.8 (2.0–5.8)	–	0 (±0)	No tumour
Choline, methionine, folate	No	Mild	20.5 (17.0–21.8)	Normal	0.6 (±0.2)	No tumour
No deficiency	No	No deficiency	21.2 (19.5–22.8)	Normal	0.2 (±0.2)	No tumour

MRI, magnetic resonance imaging.

levels were lower in gastrectomized rats than in methyl- and vitamin B_{12}-deficient diet fed animals (56). In fact, in our experience, a vitamin B_{12}-deficient diet was not efficient to reach a vitamin B_{12} deficiency with a dramatic decrease of plasma B_{12} levels. Disappearance of gastric intrinsic factor after total gastrectomy is thought to lead to vitamin B_{12} deficiency in rats by diminution or absence of vitamin B_{12} absorption (57–60). We found that i) vitamin B_{12} plasma levels were similar in rats that underwent total gastrectomy and concomitant ileal resection than in those with only total gastrectomy; ii) ileal resection alone was not efficient enough to provide low plasma levels of vitamin B_{12} (23). This indicated that an intrinsic factor was likely produced in the gastric wall and not in duodenum or jejunum as proposed by some authors (61). This also indicated that intrinsic factor-vitamin B_{12} receptor expression was likely not restricted to the ileum in rats (59, 62). An advantage of using a surgical model to reach vitamin B_{12} deficiency is to obtain a more dramatic decrease in B_{12} plasma levels and to avoid substances such as nitrous oxide or other drugs that do produce B_{12} deficiency (12, 63) but for which it is impossible to exclude interference with liver tumour carcinogenesis by direct effects.

The main disadvantage of using total gastrectomy is that this operation is a major procedure in rats. Immediate postoperative mortality has been evaluated from 20 to 50% (59, 64, 65). Furthermore, a body weight loss occurred in survivor rats (65, 66). Although survival can be as long as 3 to 10 months after gastrectomy in a single rat (67, 68), we consider that gastrectomy is not an effective way to get long term B_{12}-deficient survivor rats. Thus, gastrectomized rats do not survived long enough to permit the evaluation of liver tumourigenicity (23) (Table 2).

Deficiencies of choline, as well as methionine to a lesser degree, are the major dietary nutrients governing the severity of methyl-deficiency and liver carcinogenesis (hepatocellular carcinoma) in rat models. However, the composition of the methyl-deficient diets has been quite variable in different studies with respect to i) type and amount of protein as the major sources of methionine, ii) the levels of methionine and choline in the diets, and iii) the presence and amounts of other important components including vitamin B_{12} and folic acid (1). Consequently, differences in the magnitude of the effect on carcinogenesis among methyl-deficient diets have been observed and it is difficult to compare the intensities of the methyl-deficiencies and liver carcinogenicity in the different studies (31). Dietary vitamin B_{12} is crucial for carcinogenicity of a methyl-deficient diet. Indeed, the carcinogenicity of a methyl-deficient diet is much higher in the presence of vitamin B_{12}, compared to a methyl-deficient diet without B_{12} (48).

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