ABSTRACT  In the early 1930s, the group of Banting and Best showed that the choline moiety of lecithin was responsible for the prevention of the fatty livers produced in pancreatectomized dogs treated with insulin. This was the first study linking abnormal methyl metabolism with disease. Since then, deficiencies of each of the four essential dietary sources of methyl groups (choline, methionine, vitamin B-12 and folic acid) have been associated with increased risk of a number of diseases. Choline-deficient diets were shown to enhance liver tumor formation in rats, and such diets frequently were found to lead to atherosclerosis. Although methionine deficiency per se was not extensively studied in vivo, its metabolic antagonist ethionine did cause liver cancer and pancreatic toxicity in rodents. Deficiencies of vitamin B-12 and of folic acid have long been shown to cause neurological disturbances and birth defects both in humans and in experimental animals. In 1969 inborn errors of metabolism leading to the accumulation of the demethylated metabolite of methionine, homocysteine, were proposed as contributing to the early onset of atherosclerosis. Before 1990, numerous studies described the abnormal methylation of DNA in tumors and transformed cells. Less frequently investigated, however, were the exogenous and endogenous agents leading to such abnormal methylation. These included genetic variants among rodent strains and the methyl-deficient diets that caused liver cancer. In addition, several chemicals, particularly carcinogens, were shown to alter DNA methylation. The possible links between chemically induced alterations in DNA methylation and development of other diseases were little explored. However, by 1990, a chain of causality had been established in experimental carcinogenesis linking dietary methyl deficiency with methyl insufficiency in vivo, as well as with the abnormal methylation of DNA and of specific genes. Also during this period, the diminished activity of the enzyme methylenetetrahydrofolate reductase (EC 1.5.1.20), which is responsible for the actual de novo synthesis of methyl groups, was shown to be associated with increased risk of developing atherosclerosis, neurological disorders and birth defects. The exponential rise in studies on methyl metabolism and DNA methylation since then enables us to examine here the extent to which the mechanisms by which abnormal methylation processes seem to exert their toxic effects in one disease may be applicable to other pathologies.  J. Nutr. 132: 2336S–2339S, 2002.

KEY WORDS: ∙ diet ∙ carcinogenesis ∙ DNA hypomethylation ∙ toxicology ∙ S-adenosylmethionine

Between 1930 and 1932, scientists in the laboratory of Banting and Best (reviewed in ref. 1) discovered that the choline moiety of lecithin was responsible for the prevention of the fatty livers produced in pancreatectomized dogs treated with insulin. This set of findings provided the first evidence for the essentiality of dietary methyl donors in disease prevention. It is also interesting that their studies linked two diseases that are discussed elsewhere in this supplement: hepatotoxicity and diabetes. Since then, several other pathologies have been shown to be associated with deficiencies of one or more of the essential dietary sources of methyl groups: choline, methionine, folic acid and vitamin B-12.

The long-term administration of diets deficient in choline was seen to cause liver cancer in rats (2,3) (Fig. 1). Even though such diets were later found to be contaminated with aflatoxin, the choline deprivation was still seen to play an essential role in the formation of the tumors (4,5). Ethionine, a methionine antagonist, was found to be a liver carcinogen in rats (6). One of its major metabolites, S-adenosylethionine (SAE)3 is the ethyl analogue of the body’s chief physiological

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3 Abbreviations used: AAF, 2-acetylaminofluorene; DDT, dichlorodiphenyldichloroethane; Dnmt, DNA methyltransferase; SAE, S-adenosylethionine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.
methyl donor S-adenosylmethionine (SAM) and is an effective inhibitor of many methylation reactions, including DNA methylation (6,7). In addition to its hepatocarcinogenic effects, ethionine exhibits considerable toxicity toward the pancreas (6).

Since at least 1969, folate deficiency has been associated with spina bifida in humans (8). Similarly, the hematological and neurological complications arising from deficiencies of vitamin B-12 have long been known and well described (9). Fluctuations in the supply of methyl groups can influence the formation and excretion of homocysteine (Fig. 1). The homocystinemia caused by inborn errors of metabolism with low levels of methionine synthase (5-methyltetrahydrofolate–homocysteine S-methyltransferase EC 2.1.1.13) or cystathionine-β-synthase (EC 4.2.1.22) resulted in premature atherosclerosis (10). A related observation, of which we shall read much in subsequent papers, is the association of elevated blood homocysteine with atherosclerosis (10). A related observation, of which we shall read

FIGURE 1 The metabolic interrelations among the dietary sources of methyl groups and related compounds.

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RESULTS

Dietary methyl deficiency and liver cancer. Of the various pathologies that seem to result from methyl group insufficiency, cancer has provided our greatest knowledge about mechanisms. After the initial discoveries by Salmon and Copeland (2,3) of the hepatocarcinogenicity of choline-deficient diets, subsequent studies by Paul Newberne (4,5) showed that the liver tumors caused in rats by choline deficiency were associated with the presence of aflatoxin in the peanut meal used in these diets. Newberne continued these studies at the Massachusetts Institute of Technology in the late 1960s and 1970s, mainly in collaboration with Dr. A. Rogers. Their results showed that diets moderately low in methyl donors (termed “low-lipotrope” in those studies) exhibited cocarcinogenic activity with several liver carcinogens (1). These studies were extended in the 1970s by Lombardi and Shinozuka (11,12), who demonstrated that diets that were solely deficient in choline not only exhibited cocarcinogenic activity with several rat liver carcinogens but also served as efficient promoters in this organ. Finally, most studies with the methyl-deficient diets focused on liver carcinogenesis. However, several other lesions were also noted. These included: hemorrhagic kidneys, teratogenicity and atherosclerosis (1,13).

A prospective chain of causality by dietary methyl deficiency. In 1983, using an amino acid-defined diet, Poirier demonstrated that a severe methyl deficiency produced liver cancer in rats even in the absence of any additional carcinogen (14); less marked deficiency of either methionine or choline exhibited good liver tumor promoting activity. The hepatocarcinogenic activity of diets that were solely deficient in choline was also described in other studies (15,16). Studies on the increase in SAM levels and the increase in SAH levels in the livers of rats fed methyl-deficient diets provided evidence for a causative role by abnormal methyl metabolism in the cancer formation by such diets. Chronic feeding of lipotrope-deficient diets and of methionine- and choline-deficient diets both led to marked decreases in the hepatic SAM levels in sensitive animals (17,18). However, feeding these diets to female rats and C3H mice, both of which are insensitive to the tumorigenic effects of the respective methyl-deficient diet, had little or no effect on hepatic SAM levels (17,19). In addition, the tumorigenic activities of the amino acid-defined, methionine- and choline-deficient diets were found to be proportional to the corresponding decreases in the hepatic SAM/SAH ratios produced by the diets (20).

The methyl-deficient diets also altered DNA methylation. The long-term administration of diets deficient in the amino acids methionine and choline led to global hypomethylation of hepatic DNA (20), as well as to hypomethylation of specific oncogenes (21,22). Oncogene hypomethylation was accompanied by increased expression (23). Later studies showed that the chronic feeding of methyl-deficient diets led to abnormal methylation and facilitated mutations of the p53 tumor suppressor gene (24–26). Isoelectric focusing showed the presence of a new form of DNA methyltransferase (Dnmt; EC 2.1.1.73) activity in the liver tumors of rats fed the methionine- and choline-deficient, amino acid-defined diet (27), which could possibly explain the alterations in DNA methylation produced by the diet. The new peak of activity demonstrated a higher ratio of de novo-to-maintenance DNA methyltransferase activity, as well as an altered substrate specificity. It thus had the capacity to cause both global hypomethylation and specific site hypermethylilation (27). Additional dietary evidence that abnormal DNA methylation plays a causative role in hepatocarcinogenesis by dietary methyl deprivation was provided by the observation that caloric restriction greatly diminished the rate of loss of hepatic DNA methyl groups and prevented the formation of altered hepatic foci, commonly recognized as preneoplastic lesions, in rats fed a carcinogenic, methyl-deficient diet (28).

Chemical carcinogenesis and methyl group deficiency. Two categories of chemical agents provide evidence that chemical carcinogenesis occurs in part through abnormal methylation processes: 1) classical carcinogens and tumor promoters and 2) the antimetabolites of methylation reactions. To illustrate this point, only a few examples need be cited in each category. The first includes: 2-acetylaminofluore (AAF), diethylnitrosamine, phenobarbital, dichlorodiphenyltrichloro-
ethane (DDT), nickel (Ni), arsenic (As), zinc (Zn) deficiency and methypyrilene; each of these agents alters either SAM or DNA methylation levels (29–34). In fact, AAF was the first carcinogen whose administration under an in vivo carcinogenic regimen was shown to cause DNA hypomethylation (29). The tumorigenic activities of AAF, diethylnitrosamine, DDT and phenobarbital have all been shown to be enhanced by dietary methyl group deficiency or inhibited by supplemental methyl donors (30).

The antimetabolites of methylation, ethionine and azacitidine provide additional evidence for a causative role of abnormal methylation in carcinogenesis. As noted earlier, the methionine antagonist ethionine causes liver cancer in rats and mice (6,35,36). Both ethionine and its major metabolite SAE transform liver epithelial cells in culture (37); both inhibit SAM-dependent DNA methylation (7,38). Azacitidine is a widely recognized inhibitor of DNA methylation (39); although it is not widely classified as a carcinogen, it does enhance the formation of leukemia (40) and lung tumors (41) in mice, testicular and liver cancer (42) in rats and transforms mouse fibroblasts in culture (43). Further evidence in support of the role of abnormal methylation in carcinogenesis was provided by the studies of Feo and colleagues (44). They showed that the administration of exogenous SAM to phenobarbital-treated rats inhibited both the formation of altered foci and the hypomethylation of DNA induced by phenobarbital. Both chemopreventive effects of SAM were abrogated by the simultaneous administration of azacitidine, thus demonstrating the importance of abnormal SAM-dependent DNA methylation in liver tumor promotion by phenobarbital.

**Genetics and methylation in experimental carcinogenesis.**

Strain differences in experimental carcinogenesis studies have also contributed to a mechanistic understanding of the role of methyl metabolism in cancer formation. Choline deprivation caused altered hepatic foci in male F344 rats, but not in male PVG rats (45). Methyl-deficient diets cause liver tumors in male B6C3F1 mice, but not in the C3H strain (1,46). Finally, both rats and mice exhibit significant strain differences to the carcinogenic activity of ethionine (6,35).

Strain differences in mice have been used by the group of J. Goodman (47–49) to show strong associations between tumor formation and global DNA or oncogene hypomethylation in three related strains of mice: the inbred parental C3H and C57BL strains and the hybrid B6C3F1 strain (Table 1). The table illustrates that one parental strain, the C3H, which has a high background incidence of liver cancer, has specific hypomethylated loci in the H-ras and raf protooncogenes. The second parental strain, the C57BL, which has a low background incidence of liver cancer, has both oncogene loci methylated (Table 1). The B6C3F1 hybrid, which has an intermediate but significant incidence of spontaneous liver cancer, is similar to the C3H mouse in having these oncogene sites hypomethylated. Phenobarbital treatment, which increases the incidence of liver tumors in the C3H and B6C3F1 mice, also causes gene and global DNA hypomethylation in these strains; conversely, phenobarbital feeding causes no comparase changes in DNA methylation, nor does it exert appreciable tumorigenicity in the livers of C57BL mice. Dietary methyl deficiency, on the other hand, increases both tumor incidence and global DNA hypomethylation in the B6C3F1 mouse but not in the C3H mouse (Table 1). Thus, within this limited series of studies, a good association was seen between both global and specific gene hypomethylation and sensitivity to liver tumor formation.

The impact of genetic variability on methylation and cancer susceptibility in experimental animals has its parallels in humans. The association between atherosclerosis and genetic defects in two enzymes leading to the accumulation of homocysteine has already been described (10). That other genetic differences impacting directly upon methyl metabolism might play a role in the etiology of human disease was recognized by the early 1990s. Genetic defects resulting in diminished activity of the enzyme methylenetetrahydrofolate reductase were associated with increased risk of heart disease (30) and of neurological disturbances (51). The subsequent speakers at this workshop described in great detail the many advances that have been made on the number of diseases associated with abnormal methylation processes, as well as on the mechanisms underlying the dietary and genetic causes of such pathologies. Because of the limitations of time, the chemical causes will be evaluated in detail elsewhere.

By 1990, much evidence had been accumulated linking the abnormal metabolism of methyl donors with the development of several different pathological effects. Although cancer was the mostly widely investigated of these diseases, they also included heart disease, neurological disturbances, birth defects, aging and liver and pancreatic toxicity. Abnormal DNA methylation seems to lie at the core of many of these pathologies.

**LITERATURE CITED**


**TABLE 1**

<table>
<thead>
<tr>
<th>Cause</th>
<th>Parameter</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous</td>
<td>Tumor formation</td>
<td>C3H, B6C3F1, C57BL</td>
</tr>
<tr>
<td></td>
<td>H-ras hypomethylation</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Raf hypomethylation</td>
<td>+</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>Tumor formation</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Global DNA hypomethylation</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Raf hypomethylation</td>
<td>+</td>
</tr>
<tr>
<td>Methyl deficiency</td>
<td>Tumor formation</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Global DNA hypomethylation</td>
<td>+</td>
</tr>
</tbody>
</table>

1. +, strain is susceptible to tumor formation or to DNA hypomethylation under the treatment described; –, strain is relatively resistant to the treatment described.
2. From refs. 1, 19, 46–50.