



Irreversible global DNA hypomethylation as a key step in hepatocarcinogenesis induced by dietary methyl deficiency

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Abstract

Dietary methyl group deprivation is now well recognized as a model of hepatocarcinogenesis in rodents. In the present study, we examined the effects of feeding a methyl-deficient diet followed by a methyl-adequate diet on the extent of methylation of liver DNA and on the formation and evolution of altered hepatic foci. Male F344 rats were fed a methyl-deficient diet for 9, 18, 24, and 36 weeks, followed by re-feeding a methyl-adequate diet for a total of 54 weeks. Similar to previous findings, the methyl-deficient diet resulted in decreased levels of *S*-adenosylmethionine (SAM), SAM/SAH ratios, and global DNA hypomethylation. Feeding the methyl-adequate diet restored the liver SAM levels and SAM/SAH ratios to control levels in all experimental groups. In contrast, re-feeding the complete diet restored DNA methylation to normal level only in the group that had been fed the methyl-deficient diet for 9 weeks; in animals exposed to methyl deprivation longer, the methyl-adequate diet failed to reverse the hypomethylation of DNA. Liver tissue of rats exposed to methyl deficiency for 9, 18, 24, or 36 weeks was characterized by the persistent presence of placental isoform of glutathione-*S*-transferase (GST π)-positive lesions despite re-feeding the methyl-adequate diet. The persistence of altered hepatic foci in liver after withdrawal of methyl-deficient diet serves as an indication of the carcinogenic potential of a methyl-deficient diet. Substitution of the methyl-deficient diet with complete diet failed to prevent the expansion of initiated foci and restore DNA methylation in animals exposed to deficiency for 18, 24, or 36 weeks. The association between DNA hypomethylation and expansion of foci suggests that stable DNA hypomethylation is a promoting factor for clonal expansion of initiated cells. These results provide an experimental evidence and a mechanistic basis by which epigenetic alterations may contribute to the initiation and promotion steps of carcinogenesis. Published by Elsevier B.V.

Keywords: Methyl-deficient diet; Methyl-adequate diet; DNA hypomethylation; Rat hepatocarcinogenesis

Abbreviations: SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; MD, methyl-deficient; MA, methyl-adequate; GST π , placental isoform of glutathione-*S*-transferase; DNMT1, maintenance DNA methyltransferase

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1. Introduction

The evidence accumulated in recent years shows the importance of diet as a major factor in cancer development. It is well documented that deficiencies of the major dietary sources of methyl groups—methionine, choline, folic acid, and Vitamin B₁₂, are sufficient to induce liver tumor formation in male rats and certain mouse strains [1–5]. The methyl-deficient model of endogenous carcinogenesis is unique: in that dietary omission rather than the addition of chemical carcinogens leads to tumor formation [2,5]. The biochemical and molecular events predisposing to cancer in this model result from chronic metabolic stress and may provide an ideal model system to study progressive alterations that occur during carcinogenic process [3,4]. It has been shown that even after a relatively short exposure of rats to a choline-devoid diet (10–12 weeks), the liver contains initiated cells that are capable of full evolution to cancer even in the absence of active promotion [5,6]. Previous experiments of rodent methyl deficiency *in vivo* have shown that such diets lead to rapid fat accumulation in the liver, increased lipid peroxidation, necrotic and apoptotic cell death, increased cell proliferation, depletion of intracellular methyl group pools, the imbalance of deoxynucleotide pool resulting in uracil incorporation into DNA, DNA strand breakage, and increased genome-wide and gene-specific hypomethylation [7–11]. Any or all of these factors may contribute to the hepatocarcinogenic effects of the methyl-deficient diet. Additionally, male rats fed this diet have a reduced content of hepatic *S*-adenosyl-*l*-methionine (SAM) and elevated level of *S*-adenosylhomocysteine (SAH), a potent inhibitor of cellular methylation reactions [12]. This observation indicates that a methyl-deficient diet induces stress on the methyl donor pool and the folate-dependent resynthesis pathway of methionine from homocysteine. While it is clear that dietary methyl deprivation causes liver cancer in rodents, the mechanism by which such cancer is induced and, more specifically, the roles played by SAM insufficiency and DNA hypomethylation remain to be determined.

The results of studies conducted by other investigators and ourselves show that alteration in methylation of hepatic DNA is one of the earliest key steps that may contribute to the carcinogenic effect of methyl-deficient diet [8,10,11]. In general, methyl deficiency

induces global and gene-specific DNA hypomethylation, which is accompanied by a paradoxical increase in DNA methyltransferase activity and expression [10,11]. Despite the fact that global DNA hypomethylation was the first epigenetic alteration identified in cancer cells [13,14], most of the research in the field of cancer epigenetic has been focused on the role of increased methylation of specific gene promoters for initiating or enforcing silencing of tumor suppressor genes [15,16]. The mechanisms responsible for loss of DNA methylation and functional importance of DNA hypomethylation during carcinogenesis are unclear. Several possibilities that may contribute to the development of DNA hypomethylation have been proposed, including reduction of methylation capacity because of intracellular depletion of SAM, the inhibitory effect of SAH on DNA methyltransferases, the presence of unrepaired lesions in DNA that interfere with the methylation ability of DNA methyltransferases, and the inability of mammalian maintenance DNA methyltransferase (DNMT1) to methylate double-stranded unmethylated CpG sites [9]. A major gap in the understanding of the role of DNA methylation dysregulation in carcinogenic process is the lack of knowledge about specific alterations in cytosine methylation that may be mechanistically related to neoplastic transformation and the precise timetable of epigenetic alterations occurring between the transitions of a normal cell through intermediate tumorigenic stages to a tumor cell [17,18].

The results of previous studies indicate that feeding of a methyl-deficient diet for as little as 3–4 weeks rapidly induces loss of cytosine methylation [8–10]. The first morphological evidence of the initiation process, as indicated by the altered expression of the placental isoform of glutathione *S*-transferase (GST π), was evident after 3 weeks of deficiency, by the appearance of single GST π -positive hepatocytes; after 9 weeks of deficiency GST π -positive foci were also evident [19]. These results show that the appearance of DNA hypomethylation precedes the formation of initiated foci in hepatocarcinogenesis induced by methyl group deprivation. It has been shown that feeding animals a control diet containing adequate levels of methionine, choline, folic acid, and Vitamin B₁₂ can restore the DNA methylation pattern produced by 3–4 weeks of methyl deficiency [10]; however, it was speculated that if a methyl-deficient diet induced the accumulation of unmethylated sites resistant to

methylation, the cumulative global and gene-specific DNA hypomethylation due to dietary methyl deficiency could also have long-term consequences with regard to susceptibility to tumorigenesis [8,10,20].

To investigate the possible role of abnormal DNA methylation in carcinogenesis by dietary methyl group deprivation, we examined the effects of feeding a methyl-deficient diet followed by a methyl-adequate diet on the extent of methylation of liver DNA and on the formation of altered hepatic foci.

2. Materials and methods

2.1. Animals, diets and tissue preparation

Male weanling F344 rats were obtained from the National Center for Toxicological Research breeding facility, housed two per cage in a temperature-controlled (24 °C) room with a 12 h light/dark cycle and given ad libitum access to water and NIH-31 pelleted diet (Purina Mills, Richmond, IN). At 4 weeks of age, the rats (body weight 50 g) were allocated randomly to receive either a diet low in methionine (0.18%) lacking in choline and folic acid (methyl-deficient), or a control diet (methyl-adequate) supplemented with 0.4% methionine, 0.3% choline, and 2 mg/kg folic acid (Dyets Inc., Bethlehem, PA) [9]. Diets were stored at 4 °C and given ad libitum with biweekly replacement.

2.2. Feeding protocol

Male F344 rats were divided into five groups, one control and four experimental groups. The rats from the experimental groups were maintained on the methyl-deficient diet for 9, 18, 24, and 36 weeks, followed by feeding a methyl-adequate diet with sufficient content of methionine, choline, and folic acid. Body weights and food consumption were recorded weekly. No significant differences in the body weights between the diet groups were recorded. Four rats per group were sacrificed at 9, 18, 24, or 36 weeks of methyl deficiency. The remaining 10 rats per group were sacrificed at the end of the experiment (54 weeks). The livers were excised, frozen immediately in liquid nitrogen, and stored at –80 °C for subsequent determination of SAM and SAH concentrations, and DNA isolation.

2.3. Determination of SAM and SAH concentrations

The determination of SAM and SAH content in liver tissue extracts was performed by HPLC method as previously described [21].

2.4. Determination of total percent of unmethylated CCGG sites in DNA by cytosine extension assay

Genomic DNA was isolated from frozen liver by digestion with proteinase K, followed by standard phenol/chloroform extraction and ethanol precipitation. A radiolabeled [³H]-dCTP extension assay was used to evaluate the level of global DNA methylation as described previously [22]. Briefly, 1 µg of genomic DNA was digested with 20 U of methylation-sensitive *HpaII* restriction endonuclease (New England Biolabs, Beverly, MA) for 16–18 h at 37 °C. A second DNA aliquot (1 µg) was digested with methylation-insensitive isoschizomer *MspI* which cleaves CCGG sites in DNA regardless of CpG methylation status. Undigested DNA served as a background control. The single nucleotide extension reaction was performed in a 25 µl reaction mixture containing 1.0 µg DNA, 1 × PCR buffer II, 1.0 mM MgCl₂, 0.25 U AmpliTaq DNA polymerase, 0.1 µl of [³H]-dCTP (57.4 Ci/mmol) and incubated at 56 °C for 1 h. Samples were applied to DE-81 ion-exchange filters and washed three times with 0.5 M Na-phosphate buffer (pH 7.0) at room temperature. The filters were dried and processed for scintillation counting. The [³H]-dCTP incorporation into DNA was expressed as mean disintegrations per minute (dpm) per µg of DNA after subtraction of the dpm incorporation in undigested samples (background). The absolute percent of double-stranded unmethylated CCGG sites was calculated as described previously [20].

2.5. Immunostaining for glutathione S-transferase-placental form (GSTP)

Formalin-fixed, paraffin-embedded liver sections were processed for immunohistochemical detection of GSTP using the biotin/extravidin/peroxidase detection system (Sigma, St. Louis, MO). Primary antibodies (rabbit polyclonal anti-human GSTP; DAKO,

Carpinteria, CA) were used at 1:1200 dilutions. Liver sections were incubated with biotinylated goat anti-rabbit Ig antibodies, followed by extravidin-conjugated horseradish peroxidase. Staining was developed with diaminobenzidine substrate, and sections were counterstained with hematoxylin and analyzed by using OPTIMAS Imager-Acquiring System.

2.6. Statistical analysis

Results are presented as mean \pm S.D. Statistical analyses were conducted by two-way ANOVA using standard software.

3. Results

3.1. Level of SAM and SAH in liver

Feeding the methyl-deficient diet resulted in a rapid, significant decline in liver SAM levels (Fig. 1A). After only 9 weeks of methyl deficiency, levels of hepatic SAM in rats were decreased by 70% as compared to controls. The exposure of animals to methyl-deficient diet for longer periods did not lead to further reduction of liver SAM content (Fig. 1A), which remained at the 50–60% depleted levels. In contrast, the levels of SAH in the livers of the methyl-deficient rats did not change significantly. The depletion of SAM in the methyl-deficient animals resulted in a significant decrease in SAM/SAH ratios—an important predictor of cellular methylation capacity. Re-feeding the methyl-adequate diet restored to normal both the liver SAM contents and the hepatic SAM/SAH ratios in all groups (Fig. 1B).

3.2. DNA methylation status

To determine the absolute percent of methylated *HpaII* sites in DNA, we used the *HpaII/MspI*-based cytosine extension assay that measures the proportion of CCGG sites that had lost methyl groups on both the strands [22]. Feeding the methyl-deficient diet resulted in rapid, substantial, and progressive hypomethylation of hepatic DNA (Fig. 2). The percent of unmethylated CCGG sites in DNA isolated from the livers of rats fed with the deficient diet for 9 weeks was 60% higher than in DNA from the livers of animals maintained on the control diet. The number of unmethylated CCGG

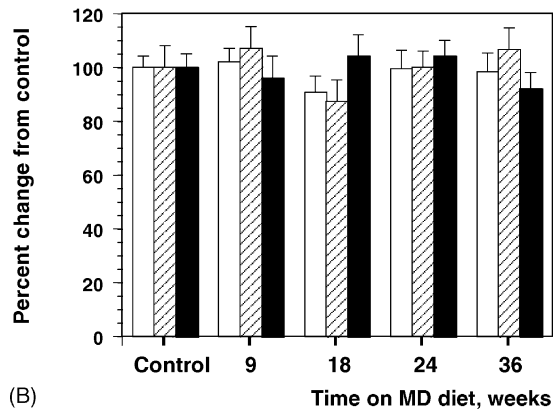
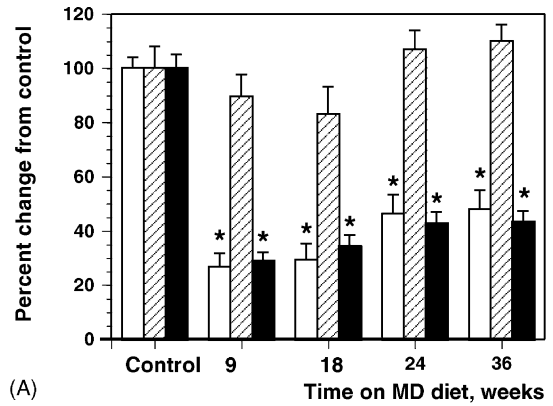


Fig. 1. Level of SAM, SAH, and SAM/SAH ratio in liver of F344 rats during methyl deficiency (A), and after re-feeding methyl-adequate diet (B): (□) SAM; (▨) SAH; (■) SAM/SAH ratio. Values significantly different from control (* $p < 0.001$).

sites in hepatic DNA after 18, 24, and 36 weeks of deficiency was 110–120% higher than in the corresponding control. Re-feeding the deficient animals the methyl-adequate diet led to the normalization of DNA methylation in F344 rats exposed to methyl deficiency for 9 weeks, but failed to reverse DNA hypomethylation in animals maintained on the methyl-deficient diet for 18, 24, or 36 weeks (Fig. 2). Even in rats fed the methyl-deficient diet for 9 weeks only, the DNA remained hypomethylated at least 3 weeks more before returning to normal levels following reinstatement of the animals to the methyl-adequate diet (data not shown). In rats exposed to methyl deficiency for longer time (more than 18 weeks), DNA remained in the hypomethylated state to the end of the experiment despite feeding animals with the methyl-adequate diet.

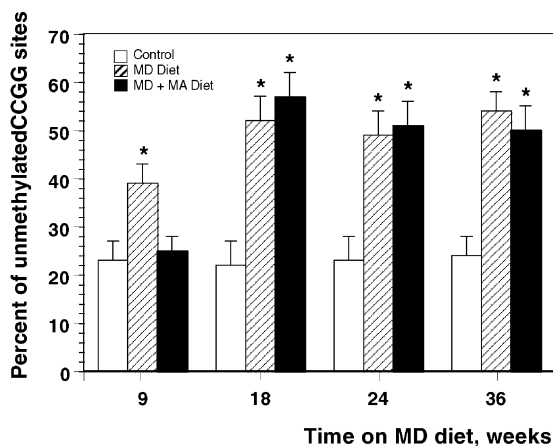


Fig. 2. Level of global DNA methylation in liver of F344 rats fed methyl-deficient (MD) diet and after re-feeding methyl-adequate diet. Values significantly different from control (* $p < 0.001$).

3.3. Analysis of GST π -positive lesions

Hepatocarcinogenesis in rodents induced by a variety of agents, including the methyl-deficient diet, is characterized by the appearance of foci of enzyme-altered (initiated) cells which can be detected by immunostaining for γ -glutamyltranspeptidase (γ -GT) or GST π . In the present study, we used immunohistochemical detection of GST π expression in liver as a more sensitive marker for carcinogenesis than γ -GT. In order to determine the significance of alterations induced by the methyl-deficient diet in regard to carcinogenesis, we looked for the presence of enzyme-altered lesions in liver tissue at the end of the experiment after re-feeding with the methyl-adequate diet. Control liver tissue did not stain for GST π . Feeding animals with the methyl-deficient diet resulted in the formation of altered hepatic foci that existed in the liver tissue even when deficient diet was substituted by diet with adequate level of methyl donors (Table 1). Even

the short exposure (9 weeks) of animals to methyl deficiency resulted in the formation of significant number of GST π -positive foci. Feeding methyl-deficient diet for 18 weeks or longer resulted in continuing increase in the number and especially in the area of GST π -positive lesions.

4. Discussion

The present study demonstrates that feeding the F344 rats a methyl-deficient diet for 36 weeks, followed by re-feeding with a complete methyl-adequate diet produced three major effects: (1) a rapid, but completely reversible decrease in hepatic SAM levels; (2) global hypomethylation of liver DNA that was reversible after 9, but not after 18 weeks or longer of methyl deficiency; and (3) the formation of persistent altered hepatic foci at all investigated time-points.

The decreased level of hepatic SAM was reported in the previous studies with varied composition of methyl-deficient diets [12]. SAM is an essential and key component for cellular transmethylation reactions, including DNA methylation. Decrease in intracellular levels of SAM correlates directly with DNA hypomethylation [8,23,24]. Indeed, a significant drop in hepatic SAM concentration led to hypomethylation of DNA in F344 rats fed the methyl-deficient diet. The reduction of hepatic SAM content resulted in a diminished SAM/SAH ratio and an upsurge of the SAH/SAM ratio. Previous studies established the association between decreased SAM/SAH ratio and DNA hypomethylation as an early feature of hepatocarcinogenesis induced by methyl deficiency [12,24]. The present study offers more support for the reliance of the normal pattern of DNA methylation in somatic cells on the SAM/SAH ratio. Alteration of any of these components results in the appearance of aberrant DNA methylation pattern.

Table 1
GST π -positive lesions in liver of methyl-deficient rats after re-feeding methyl-adequate diet

Time on MD diet (weeks)	Number of rats	Area of GST π -positive lesions (mm ²)	Area of GST π -positive lesions (% from total area)
Control	6	0	0
9	10	0.23	0.24
18	10	1.45	2.11
24	10	1.02	1.61
36	10	1.09	1.68

Determination of the role of abnormal DNA methylation in carcinogenesis induced by methyl group deficiency requires knowledge of specific alterations in DNA cytosine methylation that are mechanistically related to neoplastic transformation, as well as of the sequence of epigenetic alterations occurring in the transition from normal to the tumor cell [18]. In the present study, we examined the reversibility of DNA hypomethylation produced during hepatocarcinogenesis by methyl group deficiency. Re-feeding the methyl-adequate diet restored the DNA methylation to normal level only in the group that had been fed the methyl-deficient diet for 9 weeks. In animals exposed to methyl deprivation for 18, 24, or 36 weeks, the methyl-adequate diet failed to reverse the hypomethylation of DNA. In our previous study, we have shown the accumulation of double-stranded unmethylated sites in early preneoplastic liver tissues despite the increased DNA methyltransferase activity [20]. The failure of methyl-adequate diet to restore methylation status of DNA even with the complete normalization of hepatic SAM levels provides evidence for the importance of DNA hypomethylation in the carcinogenic process. One of the possible mechanisms of the observed irreversible DNA hypomethylation in preneoplastic liver may be related to the inability of mammalian maintenance DNA methyltransferase to methylate DNA containing significant number of unmethylated CpG sites [20,25].

It has been suggested that hypomethylation of DNA may be one of the key events in the initiation of carcinogenic process [26,27]. In general, factors that act as initiators for neoplastic cell transformation require stable changes in the genotype or phenotype of the affected cells [28]. To the contrary, promoters of tumor development provide only support for the expansion of initiated cells [28–30]. To determine the possible role of alterations in DNA methylation in regard to the carcinogenic process, we evaluated the evolution of GST π -positive preneoplastic lesions in liver after re-feeding methyl-deficient rats diet with adequate level of methyl donors. This approach clearly allows discrimination between reversible and persistent lesions in hepatocarcinogenesis because only lesions that persist after removal of carcinogen are regarded as preneoplastic or neoplastic in nature [28,30]. In animals exposed to methyl deficiency for 18, 24, or 36 weeks, liver tissue was characterized by the substantial presence

of GST π -positive lesions despite re-feeding the animals methyl-adequate diet. As we have pointed out earlier, in these groups DNA remained hypomethylated after feeding the complete diet. Unexpectedly, we detected the persistence of GST π -positive lesions in livers of rats exposed to methyl deficiency for 9 weeks only, although DNA methylation returned to control level. The accumulated evidence suggests that increased expression of GST π is a sensitive marker for initiated cells and represents precursor lesions, which are causally related to the carcinogenic process in liver [28–31]. The persistence of altered hepatic foci in liver in group of animals exposed to methyl deficiency for 9 weeks, as well as in other experimental groups, after withdrawal of methyl-deficient diet serves as an indication of the carcinogenic potential of methyl-deficient diet.

Previous studies suggested that methionine-deficient diets can serve as effective promoting agents in multistage hepatocarcinogenesis [32]. In the present study, the substitution of the methyl-deficient diet with complete diet prevented the expansion of average total area of GST π foci and restored DNA methylation only in a group exposed to deficiency for 9 weeks. In contrast, in the liver of rats exposed to methyl deficiency for longer periods, methyl-adequate diet failed to prevent the expansion of enzyme-altered lesions. The results suggest that biological and biochemical changes associated with the early stages of hepatocarcinogenesis occurred between 9 and 18 weeks of methyl deprivation. For the maintenance of promotion, the presence of at least the threshold level of the promoting factor is absolutely necessary. One of the common features of animals fed methyl-deficient diet for 18 weeks or longer is the irreversible hypomethylation of hepatic DNA. The fact that the growth of the GST π -positive lesions occurred even during re-feeding of the methyl-adequate diet indicates that DNA hypomethylation, not the methyl-deficient diet per se, may be more directly responsible for the maintenance of liver tumor promotion. Analysis of the average total area of GST π lesions, which is at least 5 times higher than in the group exposed to deficiency for 9 weeks, supports this conclusion because of the established observation that promoter-mediated clonal expansion of initiated cells leads to change in size distribution of these lesions in liver [31]. The results of the present study show that stable DNA hypomethy-

lation in tissue that undergoes carcinogenesis is an important step in the transition of normal to tumor cell and cannot be restored after formation of initiated foci. Taking into consideration that the majority of cytosine methylation in mammals resides in repetitive elements and genome-wide DNA hypomethylation largely affects transposons [33,34], stable DNA hypomethylation may have long-term consequences in carcinogenesis by leading to transposon activation and promoting chromosomal rearrangements.

The epigenetic hypothesis of cancer states that epigenetic modification can serve in two critical roles, cancer initiation and cancer progression [35]. The result of our study gives the experimental evidence to support this hypothesis and provides a mechanistic basis by which epigenetic alterations may contribute to the initiation and promotion steps of carcinogenesis.

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