Methyl deficiency causes reduction of the methyl-CpG-binding protein, MeCP2, in rat liver

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MeCP2 is a member of a family of proteins [methyl-(cytosine-guanine)CpG-binding proteins] that bind specifically to methylated DNA and induce chromatin remodeling and gene silencing. Dietary deficiency of folate, choline and methionine causes decreased tissue S-adenosylmethionine concentrations (methyl deficiency), global DNA hypomethylation, hepatic steatosis, cirrhosis and ultimately hepatic tumorigenesis in rodents. We investigated the effects of this diet on expression of MeCP2 during pre-neoplastic transformation of liver tissue. After 9 weeks, MeCP2 mRNA level was slightly higher in methyl-deficient rats compared with replete controls, while after 36 weeks, a difference in MeCP2 mRNA level was no longer observed. In contrast, MeCP2 protein level was reduced almost 2-fold in the deficient rats compared with replete controls at both 9 and 36 weeks. Conversely, a second methyl-CpG-binding protein, MBD2, showed increased levels of both message and protein at the two time points. Low MeCP2 protein in the deficient rats was associated with a low level of the co-repressor protein, Sin3a, at 36 weeks. Moreover, a known gene target of MeCP2, the tumor suppressor gene metallothionein-I, was over-expressed in the deficient rat livers at both 9 and 36 weeks, suggesting that reduction in MeCP2 may have functional consequences. Methyl deficiency also caused an increase in the ratio of long to short variants of MeCP2 transcripts. This finding suggests that reduced MeCP2 protein level is the result of a reduced rate of translation. Reduction of MeCP2 protein expression may influence the initiation and/or progression of hepatic cancer induced by methyl deficiency and may provide a useful marker of pre-neoplastic change.

Introduction
Cytosine bases, as components of cytosine-guanine (CpG) dinucleotides, are subject to methylation catalyzed by DNA methyltransferase enzymes (1). This chemical modification has important regulatory effects on gene expression. There is an inverse correlation between transcriptional activity and DNA methylation within gene-specific promoter regions (2), and transgenic mice deficient in the DNA methyltransferase enzyme, DNMT1, do not survive gestation (3). Therefore, DNA methylation is considered to be a major epigenetic control mechanism essential for normal mammalian development and cellular function.

In recent years, the components of methylation-dependent control of gene expression have been identified. The current working paradigm involves newly discovered protein factors that contain a highly conserved methyl-CpG-binding domain or ‘MBD’ (4). Five methyl CpG-binding proteins have been described (MeCP2, MBD1, MBD2, MBD3, MBD4). Among them, MeCP2, MBD1, MBD2 and MBD3 bind to methylated CpG sites and mediate methylation-dependent transcriptional repression. The fifth protein, MBD4, is believed to play a role in DNA repair.

Perhaps the best characterized of these proteins is MeCP2. Upon binding to methylated DNA, MeCP2 recruits the co-repressor protein, Sin3a, which in turn recruits the enzyme histone deacetylase. This cascade leads to histone hypoacetylation with consequent chromatin condensation and gene silencing (4,5). A major implication of epigenetic control of gene expression by MeCP2 is that it can be influenced by the methylation status of DNA. Moreover, conditions that induce aberrant DNA methylation patterns are likely to cause significant disruption of normal gene expression and cellular function, thus contributing to the pathogenesis of disease. This phenomenon is currently receiving attention as a pathophysiological mechanism underlying promotion of cancer.

Reduced dietary supply of the substrates for generation of labile methyl groups, specifically folate, choline and methionine, causes global DNA hypomethylation, hepatic steatosis, hepatomegaly and eventually hepatic cancer in rats (6). We have been exploring the hypothesis that altered expression of methyl-CpG-binding proteins may regulate the expression of tumor-suppressor genes or may play a role in the pathogenesis of hepatic cancer in this model. In the present study, we report that the hepatic level of MeCP2 protein is reduced in pre-neoplastic methyl-deficient rats. Moreover, we report that reduced MeCP2 is associated with a reduction in the co-repressor protein, Sin3a, as well as increased expression of metallothionein-I (MT-I), a known gene target for transcriptional repression by MeCP2. Last we provide data indicating that the reduction in MeCP2 protein level may result, at least in part, from a reduced rate of translation caused by an alteration in the ratio of long to short variant transcripts for this protein.

Materials and methods

Animals and diets
Weanling male F344 rats weighing 50–75 g were paired by weight. One of each pair was randomly assigned to receive a semi-purified diet deficient in folate and choline and low in methionine (0.18%), while the second of each pair received the same diet containing 2 mg/kg folate, 0.3% choline and...
0.4% methionine (Dyets, Bethlehem, PA). The rats were fed the diets for 9 and 36 weeks, then killed by CO2 asphyxiation. Their livers were excised, a portion taken for formalin fixation, and the remainder snap frozen in liquid nitrogen and stored at −80°C until analysis. The time points, 9 and 36 weeks, respectively, represent reversible and irreversible pre-neoplastic stages in the development of hepatic cancer in this model (7,8).

Morphological assessment
Microscopic sections were prepared from paraffin-fixed blocks taken from representative animals at 9 and 36 weeks. Microscopic evaluation was carried out on hematoxylin and eosin, reticulin and Masson trichrome-stained sections of this material.

Total RNA and nuclear protein preparation
Total RNA was isolated using Trizol Reagent (Life Technologies, Grand Island, NY). For nuclear extracts, nuclei were first isolated by homogenizing rat tissues in cold homogenization buffer [10 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA and EGTA, 1 mM DTT, 1% NP-40, and protease inhibitor cocktail (Sigma, St Louis, MO)], followed by microcentrifugation at 14,000 r.p.m. for 1 min. The nuclear pellets were then washed and resuspended in protein extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl 1 mM each of EDTA and EGTA, and protease inhibitor cocktail), incubated on ice for 15 min, and microcentrifuged at 11,000 r.p.m. for 5 min at 4°C. Nuclear extracts were transferred to new tubes and stored at −80°C until analysis. The concentration and integrity of each protein preparation were determined by colorimetric total protein assay (Dy; Protein Assay, Bio-Rad, Hercules, CA) and SDS-PAGE followed by Ponceau Red staining.

Semi-quantitative reverse transcriptase–PCR (RT–PCR)
Following DNase treatment, first-strand cDNA was synthesized from 2 μg total RNA digested with random hexamers by using SuperScript reverse transcriptase (Life Technologies). Several parallel PCR reactions were performed; MeCP2, MB2D, Sin3α, MT-1 and proliferating cell nuclear antigen (PCNA) were amplified to determine expression level, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as a control for RNA amount and integrity. Following cDNA synthesis, 1–2 μl of the product from each sample was used as the PCR template (0.5 μl for GAPDH). PCR reactions were performed in 50 μl reaction volumes for 30 cycles (24 cycles for GAPDH), each consisting of denaturation at 94°C for 1.5 min, annealing at 57°C for total MeCP2 and MeCP2 with long 3'-untranslated regions untranslated regions (UTR) transcripts for 50 s, and extension at 72°C for 1 min. Primer pairs were as follows: MeCP2 (total), 5'-CAG-CTG-CTA-CAG-GAT-TCC-ATG-CT-3' (sense) and 5'-TGA-TGT-CTC-TGC-TTT-GCC-GTC-CT-3' (antisense); MeCP2 (long 3'-UTR), 5'-CTT-GTG-TCT-TTT-AAA-GCA-GAG-AGC-3' (sense) and 5'-TAC-ATG-GTC-ACC-TAC-CTG-TCA-3' (antisense); MB2D, 5'-GGC-AGA-AGC-GAT-GTC-TAC-3' (sense) and 5'-CTG-GAC-GCA-CTC-CTT-GAA-3' (antisense); Sin3α, 5'-TCC-CTG-AGC-ATG-GAT-GCC-3' (antisense); MT-1, 5'-AGC-AGC-AGA-CTG-GTC-TCT-3' (antisense); GAPDH, 5'-GGT-GCT-GAG-TAT-GTC-GTG-GA-3' (sense) and 5'-TGT-GTG-GTG-ATG-GCT-GCC-3' (antisense); and PCNA, 5'-ATC-CAG-GGC-TCC-ATC-CTG-3' (sense) and 5'-CAG-AGA-GTA-AGC-TGC-3' (antisense). The cycle number for each gene was chosen when the amount of PCR product was in the exponential phase of the PCR reaction. Each RT–PCR assay was repeated several times for confirmation and assay reproducibility which was 95–99%. PCR products were analyzed on 2% agarose gels containing ethidium bromide, and signals were quantified with a Gel Doc 1000 with Quantity One Software (Bio-Rad).

Western blotting
Twenty micrograms of nuclear extracts from each sample were loaded onto an 8% SDS-polyacrylamide gel, electrophoresed in Tris–glycine–SDS running buffer, and transferred to a nitrocellulose membrane at 4°C. After blocking with 5% non-fat dry milk in Tris-buffered saline with Tween-20 (10 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Tween 20) for 1 h at room temperature, the membranes were hybridized with antibodies against MeCP2, Sin3α and MB2D (Upstate Biotechnology, Lake Placid, NY) overnight at 4°C. An anti-actin antibody (1:15 000 dilution; Sigma) was used to control for unequal loading. The membranes were then washed three times with TBS-T buffer at room temperature, followed by incubation with 1:5000 dilution of secondary HRP-conjugated antibodies (anti-rabbit for MeCP2 and Sin3α, anti-sheep for MB2D and anti-mouse for β-actin; Amersham, Piscataway, NJ) for 1 h at room temperature. The proteins were visualized with enhanced chemiluminescent detection solutions (ECL; Amersham) and exposed to ECL-hyperfilm (Amersham). Signals were quantified with a Gel Doc 1000 with Quantity One Software (Bio-Rad).

Statistical analyses
Mean gene expression levels, quantified by densitometry and normalized to GAPDH (message) or β-actin (protein) were compared between deficient and replete animals by standard t-tests. Statistically significant differences were defined as P < 0.05.

Results
Effect of methyl deficiency on hepatic morphology
Hematoxylin and eosin-stained sections of liver taken from methyl-deficient animals at 9 weeks showed marked steatosis diffusely distributed throughout the liver lobules. Cytological features were unremarkable. Reticulin and Masson’s stain showed clear evidence of reticulin and collagen fiber deposition, respectively. No evidence of regenerative nodule formation was seen. In the sections from 36-week methyl-deficient animals, the steatosis, although still present and of similar intensity, was distributed less diffusely due to the presence of numerous regenerative nodules permeating the tissue. Within the regenerative nodules, cells showed pleomorphic features with nuclear dysplasia. Several mitotic forms were identified, but there was no clear evidence of neoplasia. Both reticulin and Masson stain showed evidence of more dense fibrosis. These findings are in keeping with progressive cirrhosis typical of this model and are consistent with pre-neoplastic stages of liver injury models for hepatic tumorigenesis, in general.

Effect of methyl deficiency on hepatic MeCP2 and MB2D mRNA and protein levels
The effect of methyl deficiency on hepatic MeCP2 and MB2D mRNA levels (normalized to GAPDH mRNA) after 9 and 36 weeks was determined by semi-quantitative RT–PCR. After 9 weeks, MeCP2 message was, on average, 1.8-fold higher (P = 0.017) and MB2D message 2.3-fold higher (P < 0.001) in the deficient rats compared with replete controls (Figure 1A and B). After 36 weeks, no significant difference in MeCP2 message was detected, whereas the mean MB2D message was 1.4-fold higher in the deficient animals compared with controls (P = 0.001) (Figure 1C and D). The mean mRNA level of PCNA (an indicator of cell proliferation) was higher in the deficient rats than in controls at both time points (P ≤ 0.004) (Figure 1A–D).

To analyze whether mRNA levels corresponded with protein expression levels, western blot analyses were performed on hepatic nuclear extracts prepared from the same deficient and control tissues. After 9 weeks, despite the observed increase in mRNA, the mean MeCP2 protein level (normalized to β-actin protein) was 2.9-fold lower in the deficient rats compared with controls (P = 0.004) (Figure 2A and B). After 36 weeks, the mean MeCP2 level remained decreased in the deficient rats compared with controls (1.8-fold lower; P = 0.012) (Figure 2C and D). This pattern is contrasted with MB2D protein level, which was 1.4- and 3-fold higher, on average, in the deficient rats compared with controls after 9 and 36 weeks, respectively (P ≤ 0.032) (Figure 2A–D).

Effect of methyl deficiency on the co-repressor protein, Sin3α
After binding to methylated DNA, MeCP2 recruits the co-repressor protein, Sin3α, thus forming the MeCP2/Sin3α transcription repressor complex (5). Because of the observed changes in MeCP2 message and protein, we also determined
the effect of methyl deficiency on Sin3a message and protein. Like MeCP2, the mean hepatic Sin3a mRNA level (normalized to GAPDH) was slightly higher at 9 weeks in the deficient rats compared with controls \( (P \approx 0.048) \) (Figure 1A and B), whereas no significant difference was observed at 36 weeks (Figure 1C and D). After 9 weeks, no significant difference in Sin3a protein level (normalized to \( b\)-actin) was observed (Figure 2A and B); whereas after 36 weeks, Sin3a protein was on average 1.9-fold lower in the deficient rats compared with controls \( (P \approx 0.045) \) (Figure 2C and D).

**Effect of methyl deficiency on MT-I expression**

To determine the potential functional relevance of reduced MeCP2 protein, we investigated the effect of methyl deficiency on the expression of MT-I. The MT-I gene is known to be a target for transcriptional repression by MeCP2 (9). Accordingly, we hypothesized that MT-I expression would be increased in methyl-deficient rat liver. At both 9 and 36 weeks, the mean hepatic MT-I mRNA level (normalized to GAPDH) was 1.7-fold higher in the deficient rats compared with replete controls \( (P \leq 0.013) \) (Figure 1A–D).

**Potential mechanisms for reduced MeCP2 protein in methyl deficiency**

Two potential mechanisms to explain the observed reduction in MeCP2 protein in methyl deficiency were investigated. First, transport of MeCP2 from its site of synthesis in the cytoplasm to the nucleus might be impaired in methyl deficiency. MeCP2 is a nuclear protein and the data shown in Figure 2 represent protein levels in nuclear extracts. If transport of MeCP2 from the cytoplasm to the nucleus was impaired, there would be an accumulation of the protein in the cytoplasm. Figure 3 shows that MeCP2 protein was not detectable by western blot analysis in cytoplasmic fractions from either methyl-deficient or replete rats indicating that efficiency of transport of the protein to the nucleus was not impaired by methyl deficiency.
Secondly, we considered that the translational efficiency of MeCP2 might be affected by methyl deficiency. It was recently reported that MeCP2 has five different polyadenylation sites, which encode for mRNA species containing variable length 3′-UTRs. The longest 3′-UTR is part of an alternatively polyadenylated, 10 kb MeCP2 transcript which is differentially expressed in brain and other tissues (10). This ‘long’ MeCP2 transcript may be translated less efficiently than the ‘short’ MeCP2 transcripts (10,11). Because we found that MeCP2 protein level is reduced in methyl deficiency, we hypothesized that methyl-deficient rats might exhibit a relatively higher amount of the long MeCP2 transcript than replete controls. As shown in Figure 4, the mean level of the long MeCP2 transcript (normalized to GAPDH mRNA) was ~1.5-fold higher in the methyl deficient rats compared with the replete controls (P < 0.001). Because there was no difference in total MeCP2 mRNA between the experimental groups (Figures 1 and 4), it can be inferred from these results that the methyl-deficient rats had lower levels of the short MeCP2 transcripts compared with the controls.

Discussion

We have used the methyl-deficient rat model of hepatic carcinogenesis to study pre-neoplastic expression of methyl-CpG-binding proteins. In this purely dietary model, progressive changes in cellular methylation and gene expression during cancer development can be evaluated readily in vivo (7,8). The primary finding of the present study is that methyl deficiency causes a significant decrease in hepatic MeCP2 protein despite causing no decrease in MeCP2 mRNA. This finding is contrasted with hepatic MBD2 for which both message and
protein were increased by methyl deficiency. These changes in methyl-CpG-binding proteins are observed at an early, reversible, pre-neoplastic stage of methyl deficiency-induced carcinogenesis (9 weeks), characterized by diffuse steatosis and fibrosis, but little or no dysplasia. The changes also persist at a later, irreversible, pre-neoplastic stage (36 weeks), characterized by diffuse steatosis and fibrosis, interspersed with focal regions of nodular regeneration showing early dysplastic changes.

These findings raise two fundamental and related questions. First, what is the functional relevance of reduced MeCP2 protein in this model? As described in the introduction, the primary function of MeCP2 is transcriptional repression. In a condition that leads to reduced MeCP2 protein, such as that observed in the present study, we postulate that genes that are targets of repression by MeCP2 will become over-expressed. Consistent with this hypothesis is that MT-I, a known target of MeCP2 repression (9), was over-expressed in the methyl-deficient livers. The observed correlation will, however, need to be confirmed by further mechanistic studies to determine if the over-expression of MT-I is the direct consequence of reduced MeCP2 protein. It is important to note that over-expression of MT-I occurred despite the fact that a second methyl-CpG-binding protein, MBD2, was over-expressed in methyl-deficient livers. This strongly suggests the functions of MeCP2 and MBD2 are not redundant, as has been suggested (12), and that there may be specific gene targets for MeCP2-mediated transcriptional repression that are distinct from genes targeted by MBD2.

The second fundamental question is what causes MeCP2 protein to be reduced in methyl deficiency? In the present study, we found that the cause of reduced MeCP2 was not impaired transport of the protein from its site of synthesis in the cytoplasm to its site of function in the nucleus. We found evidence that alternate polyadenylation may play a role. Variant transcripts exist for MeCP2, designated 'long' and 'short', that differ by the lengths of their 3'-UTR regions (10). Moreover, the long MeCP2 transcript inversely correlates with MeCP2 protein expression, suggesting that the long transcript is more slowly translated than the short transcripts (11). In keeping with this concept, we found that the long MeCP2 transcript was over-represented in the methyl-deficient rats compared with replete controls. If the long MeCP2 transcript is less efficiently translated than the short transcripts, then this could explain the observed reduction in MeCP2 protein despite no decrease in total MeCP2 message.

We have also considered the possibility of increased protein degradation as a cause of reduced MeCP2 protein in methyl deficiency. One of the consequences of a methyl-deficient diet is global DNA hypomethylation (13,14). Presumably, this condition of global DNA hypomethylation affects the promoter regions of MT-I and other putative targets of MeCP2 repression. It is possible that binding to methylated CpG regions of DNA and other components of the repressor complex protects MeCP2 from proteolysis. Conversely, when DNA is hypomethylated, unbound MeCP2 may be more susceptible to proteolysis. This would account for the lower level of MeCP2 protein that we observed in methyl-deficient animals. This hypothesis remains to be tested. Another possibility is suggested by a recent study in which Sin3a was shown to protect the tumor suppressor protein, p53, from proteolysis (15). Similar to MeCP2, p53 serves as a repressor protein by a mechanism that involves recruitment of Sin3a (15). In the present study, we found Sin3a to be reduced in the methyl-deficient rats after 36 weeks. Sin3a may protect MeCP2 from proteolysis as it does p53. Thus, reduced MeCP2 protein in methyl deficiency may be the consequence of reduced Sin3a. This possibility is tempered, however, by the finding that Sin3a protein level was not reduced after 9 weeks of methyl deficiency, despite the observed decrease in MeCP2 protein level at that time point.

The impetus for the present study was the hypothesis that alterations in methyl-CpG-binding proteins, such as MeCP2, may play a role in either the promotion or retardation of hepatic carcinogenesis. Such a role for MeCP2 remains speculative. The initial pre-neoplastic effect of methyl deficiency in rodents is liver injury resulting from steatosis (16). The hepatic response to this injury is cellular proliferation and hepatomegaly. Indicative of this hyperproliferative state is the elevated level of PCNA message detected in the methyl-deficient rats. Therefore, it is possible that the observed changes in MeCP2, MBD2, Sin3a and MT-I are characteristic of proliferating pre-neoplastic cells that are a response to the induced liver injury. Because methyl deficiency ultimately leads to hepatocellular carcinoma, these changes may influence the tumorigenic process, either as promoting or retarding agents. The latter role seems more likely in that MT-I has antitumorigenic properties (9). If the reduction in MeCP2 protein is directly responsible for increased expression of MT-I, then this may be a mechanism by which the liver is protected against tumorigenic transformation in methyl deficiency. Because hepatocellular carcinomas do eventually occur in this model, the assumption is that the protection afforded by reduced MeCP2 and increased MT-I is eventually...
overcome in the face of relentless chronic liver injury. This is supported by the observations that rat hepatocellular carcinomas over-express MeCP2 and do not express MT-I (9). However, it is also probable that other genes are targets for repression by MeCP2. Some, such as p53 and p16, both of which undergo alterations of methylation status during methyl deficiency-induced hepatic carcinogenesis (7,8,13,17), have tumor suppressor functions. Others may actually promote tumorigenesis (e.g. oncogenes). Therefore, the overall influence of reduced MeCP2 on gene expression and the development of hepatic cancer in this model remains to be determined, but is likely to be the consequence of complex genetic control mechanisms. Additional issues that remain to be examined are whether or not similar alterations in methyl-CpG-binding protein expression occur in other liver injury models of hepatocellular carcinoma (e.g. carbon tetrachloride, ethionine or ethanol exposure), as well as a model of hepatic proliferation (i.e. partial hepatectomy); whether the altered expression levels are confined to specific hepatic cell types and regions; and whether the magnitude of changes in expression levels are sufficient to be physiologically relevant.

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