Biochem. J. (1984) **218**, 987–990
Printed in Great Britain

Hypomethylation of hepatic nuclear DNA in rats fed with a carcinogenic methyl-deficient diet

Mary J. WILSON, Narayan SHIVAPURKAR and Lionel A. POIRIER
Nutrition and Metabolism Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD 21701, U.S.A.

(*Received 5 December 1983/Accepted 24 January 1984*)

A progressive decrease was observed in the 5-methyldeoxycytidine content of hepatic DNA in male F344 rats fed with a hepatocarcinogenic methyl-deficient diet. The same dietary regimen resulted in altered hepatic contents of *S*-adenosylmethionine, the methyl-donating species, and *S*-adenosylhomocysteine, an inhibitor of DNA methylase. The data indicate that this carcinogenic dietary manipulation is sufficient to alter a possible regulatory process, DNA methylation.

In eukaryotic cells, the expression of genetic information appears to be associated with extents and patterns of DNA methylation (Razin & Riggs, 1980; Ehrlich & Wang, 1981; Felsenfeld & McGhee, 1982). The high fidelity with which methylation patterns are inherited (Wigler *et al.*, 1981; Harland, 1982; Stein *et al.*, 1982) suggests that permanent alterations in gene expression might result if more than one cycle of DNA replication were to occur under conditions that favoured hypomethylation. It was observed previously in our laboratory (Shivapurkar & Poirier, 1983) that the administration to rats of a methyl-deficient diet resulted in a decrease in AdoMet, the DNA methylase substrate (Sheid *et al.*, 1968), and an increase in AdoHcy, a competitive inhibitor of DNA methylase (Cox *et al.*, 1977). We therefore decided to determine whether the same dietary deficiency would produce hypomethylation of hepatic DNA. To answer this question, the extents of hepatic nuclear DNA methylation were determined in rats receiving the methyl-deficent diet for up to 22 weeks. Contents of AdoMet and AdoHcy were monitored during the same time period. The results indicate that a dietary methyl deficiency, which of itself is hepatocarcinogenic, decreases the hepatic contents of AdoMet and produces a progressive decrease in the percentage of deoxycytidine residues modified to 5-methyldeoxycytidine in hepatic DNA.

Experimental

Materials

Components of the amino acid-defined methyl-deficient and methyl-adequate diets were purchased from Teklad Test Diets (Madison, WI, U.S.A.). Diethylnitrosamine was supplied by Eastman Chemicals (Rochester, NY, U.S.A.). AdoMet, AdoHcy, proteinase K and ribonuclease A were obtained from Boehringer Mannheim (Mannheim, W. Germany). Deoxynucleoside standards were purchased from Sigma (St. Louis, MO, U.S.A.).

Abbreviations used: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine.

Animals and initiation and feeding regimen

Male weanling Fischer 344 rats (50–60g) were supplied by the Frederick Cancer Research Facility Animal Production Area. Animals were distributed into four groups (five animals/group). Two groups were injected intraperitoneally with sterile 0.9% NaCl (5.0ml/kg body wt.), and the other two with a single initiating dose of diethylnitrosamine (20mg/kg body wt.). The rats were then maintained on the complete, methyl-containing, amino acid-defined diet (diet 1) for 7 days. After 1 week, one of the groups injected with saline and one of the groups injected with diethylnitrosamine were continued on diet 1, whereas the other saline- and diethylnitrosamine-injected groups were given a diet devoid of methionine and choline but supplemented with the methionine precursor homocystine (9g/kg of diet) (diet 2). In all other respects diet 2 was identical with diet 1 (for a detailed description of the diets, see Linnell *et al.*, 1983; Mikol *et al.*, 1983). Animals were given food and water *ab libitum* throughout the experiment. At 4, 8 and 22 weeks after the injection, animals were killed by cervical dislocation, and the livers were removed, weighed and quickly placed on ice.

Isolation of nuclei, and determination of 5-methyldeoxycytidine

Livers were homogenized in ice-cold 10mM-Tris/HCl buffer, pH7.5, containing 0.32M-sucrose,

3mM-$MgCl_2$ and 0.3% Triton X-100, by using five strokes each of a loose and a tight pestle in a Dounce homogenizer. Homogenates were centrifuged at 1000*g* for 5min at 4°C. Pellets were resuspended and centrifuged several times until a white nuclear pellet was obtained. DNA was isolated as described by Marmur (1961), with modification. Nuclear pellets were digested overnight at 37°C with 100μg of proteinase K/ml in 10mM-Tris/HCl buffer, pH8.0, containing 10mM-NaCl, 10mM-EDTA and 0.5% sodium dodecyl sulphate. After digestion, 5M-$NaClO_4$ was added to a final concentration of 1M, and the protein was extracted twice with chloroform/3-methylbutan-1-ol (24:1, v/v). Then 40% (w/v) sodium acetate was added to the aqueous layer to a final concentration of 2%. DNA was precipitated with 2vol. of ice-cold 95% (v/v) ethanol, collected by centrifuging at 2000*g* for 10min and freeze-dried. DNA was dissolved in 0.05M-Tris/HCl (pH8.0)/0.1M-NaCl/0.01M-EDTA and treated overnight at 37°C with ribonuclease A which had been heated in a boiling-water bath for 10min (40μg/ml). After ribonuclease treatment, proteinase K (100μg/ml) and sodium dodecyl sulphate (final concn. 0.5%) were added and the incubation was continued for 1h. $NaClO_4$ was then added, the sample was extracted with chloroform/3-methylbutan-1-ol, and DNA was precipitated, washed and freeze-dried as above. DNA was enzymically hydrolysed as described by Kuo *et al.* (1980), and deoxynucleosides were separated and quantified by high-performance liquid chromatography with a Beckman model 324 liquid chromatograph. An Altex Ultrasphere-ODS C_{18} column (4.6mm × 25cm) was equilibrated with 0.05M-KH_2PO_4/12% (v/v) methanol. At 4min after injection of the sample, a gradient was initiated which gave a final methanol concentration of 20% (v/v) in 1min. Flow rate was 1ml/min. The A_{280} was monitored with a Beckman model 160 detector. The Altex model C-R1A integrator was calibrated with deoxynucleoside standards.

Determinations of S-adenosylmethionine and S-adenosylhomocysteine

Portions (1g) of liver were homogenized in 2vol. of ice-cold 0.1M-sodium acetate buffer, pH6.0, and protein was precipitated with 1.5vol. of 40% (w/v) trichloroacetic acid. Protein-free supernatants were washed with diethyl ether and filtered as described previously (Shivapurkar & Poirier, 1983; Hyde & Poirier, 1982). Resulting extracts were analysed by high-performance liquid chromatography isocratically with the instrumentation and column described above. Chromatographic conditions for AdoMet were 5mM-heptanesulphonic acid/30% (v/v) methanol, pH3.3, and for AdoHcy 0.05M-KH_2PO_4/8% (v/v) methanol. Flow rate was 1ml/min. Absorbance was monitored at 254nm. The integrator was calibrated with AdoMet and AdoHcy standards.

Results

It was previously shown in our laboratory that 40% of rats receiving diet 2 alone developed hepatocellular carcinomas; however, all of the animals pretreated with a single initiating dose of diethylnitrosamine, followed by feeding of diet 2, bore tumours when killed at 76 weeks (Mikol *et al.*, 1983). We therefore decided to compare extents of DNA methylation under both experimental regimens.

The content of 5-methyldeoxycytidine in hepatic nuclear DNA of animals fed on diet 2 decreased with time (Table 1). At 4 weeks, there was no detectable change relative to control values, whereas at 8 weeks the percentage of deoxycytidine residues modified to 5-methyldeoxycytidine was descreased by 6%. At 22 weeks, the decrease was more dramatic, 11–14%. Diethylnitrosamine initiation exerted no detectable effect on extent of methylation.

Chronic feeding of diet 2 resulted in a 48% decrease in the hepatic content of AdoMet at week 4, consistent with our previous observations (Shivapurkar & Poirier, 1983), and decreases of approx. 36% at weeks 8 and 22 (Table 2). In livers of rats fed on the methyl-deficient diet, AdoHcy was increased by approx. 55% relative to control values at week 4, was unchanged at week 8 and decreased by 38% at week 22. Again, diethylnitrosamine initiation exerted no effect. Many investigators have proposed that it is the AdoMet/

Table 1. *Effect of dietary methyl deficiency on 5-methyldeoxycytidine content of hepatic DNA*
The data are expressed as the percentage of deoxycytidine residues present as 5-methyldeoxycytidine (means ± S.E.M. for five animals). The data at the 8-week time point represent the average of two separate reproducible experiments.

Group	4 weeks	8 weeks	22 weeks
Diet 1 + saline	3.42 ± 0.05	3.33 ± 0.03	3.27 ± 0.04
Diet 1 + diethylnitrosamine	3.42 ± 0.06	3.33 ± 0.03	3.25 ± 0.04
Diet 2 + saline	3.32 ± 0.08	3.13 ± 0.05*	2.81 ± 0.04*
Diet 2 + diethylnitrosamine	3.52 ± 0.09	3.13 ± 0.04*	2.90 ± 0.03*

* Student's *t* test indicates a statistically significant ($P < 0.01$) difference between animals fed on diets 1 and 2.

Table 2. *Effect of dietary methyl deficiency on hepatic AdoMet and AdoHcy contents and AdoMet/AdoHcy ratio*

The AdoMet and AdoHcy values are expressed as means (nmol/g of tissue)±S.E.M. for five animals. The data at the 8-week time point represent the averages of two separate reproducible experiments.

	4 weeks			8 weeks			22 weeks		
Group	AdoMet	AdoHcy	AdoMet/AdoHcy	AdoMet	AdoHcy	AdoMet/AdoHcy	AdoMet	AdoHcy	AdoMet/AdoHcy
Diet 1+saline	96.9±5.3	23.4±0.7	4.1±0.3	86.2±2.8	22.4±1.0	3.9±0.2	87.7±2.8	20.6±2.4	4.3±0.5
Diet 1+diethylnitrosamine	95.4±5.8	21.8±1.0	4.4±0.3	76.7±2.9	21.2±0.5	3.6±0.2	85.1±0.2	20.8±1.1	4.1±0.2
Diet 2+saline	49.8±2.3*	34.7±1.8*	1.4±0.1*	56.1±1.5*	21.2±0.8	2.6±0.1*	57.7±1.5*	12.6±0.9*	4.6±0.3
Diet 2+diethylnitrosamine	50.4±0.5*	33.0±2.2*	1.5±0.1*	55.6±1.2*	22.2±0.5	2.5±0.1*	53.1±1.8*	13.5±0.9*	3.9±0.3

* Student's *t* test indicates a statistically ($P<0.01$) difference between animals fed on diets 1 and 2.

AdoHcy ratio that is important in regulating cellular methylation reactions (Kerr & Heady, 1971; Cox *et al.*, 1977; Chiang & Cantoni, 1979; Hoffman *et al.*, 1980). The most severe decrease in the AdoMet/AdoHcy ratio in animals receiving diet 2 (67%) was seen at week 4. By 22 weeks, there was no difference in this ratio between animals receiving either diet, although, as described above, AdoMet and AdoHcy contents in the two dietary categories were quite different.

A 35–50% suppression in weight gain was observed in animals fed on diet 2 compared with those on diet 1 (results not shown). Diethylnitrosamine treatment exerted little or no effect on weight gain. The decrease in body-weight gain noted in the rats fed on diet 2 was not the apparent cause of the decreased hepatic AdoMet contents, since our previous studies demonstrated that a suppression in weight gain induced by food deprivation did not affect AdoMet contents (Shivapurkar & Poirier, 1983). Relative to the body weights, the liver weights were substantially higher at all time points in animals receiving diet 2 compared with those fed on diet 1 (results not shown). Increases in relative liver weight, hepatic cell proliferation and accumulation of fat have been observed previously in animals fed on diet 2 (Shivapurkar & Poirier, 1983) and other diets deficient in methyl donors (Newberne *et al.*, 1969; Shinozuka *et al.*, 1978; Shinozuka & Lombardi, 1980; Hoover, 1983).

Discussion

The data presented here demonstrate that a dietary modification alone is sufficient to cause significant alteration of a possible regulatory process, DNA methylation. The potential permanency of the alteration is suggested by the high degree of fidelity with which DNA methylation patterns are inherited (Wigler *et al.*, 1981; Harland, 1982; Stein *et al.*, 1982). The progressive decrease in extent of DNA methylation observed in the present study is not a non-specific consequence of the cell replication seen in diet-2-fed animals, since it has been shown that even regeneration after partial hepatectomy does not detectably alter the content of 5-methylcytosine (Gama-Sosa *et al.*, 1983). Although the decreased the hepatic contents of AdoMet in diet-2-fed animals may be responsible for the production of hypomethylated DNA, other factors, such as contents of polyamines, which are thought to inhibit DNA methylase (Cox, 1979), may also be important. The effect of diet 2 on hepatic polyamine contents is not known; however, feeding of diet 2 has been shown to result in a 10-fold increase in ornithine decarboxylase activity (Mikol

& Poirier, 1981), the first enzyme in the polyamine-biosynthetic pathway.

Many chemical carcinogens, by their interaction either with DNA or with DNA methylase, have been shown to decrease the extent to which DNA is methylated (Boehm & Drahovsky, 1983, and references therein; Chan *et al.*, 1983; Cox, 1983). It has therefore been proposed that perturbations in methylation induced by chemical carcinogens may result in permanent aberrations in gene expression responsible for neoplastic transformation. It is noteworthy that the changes in extents of DNA methylation described here were observed in the liver, the only known target for the tumour-producing diet. Furthermore, previous studies indicated that contents of AdoMet and AdoHcy in the liver were more dramatically altered during administration of diet 2 than those in any other tissue examined (Shivapurkar & Poirier, 1983). It will be of interest to determine whether tissues and species insensitive to the effect of the dietary modification on DNA methylation are also insensitive to its carcinogenic effects. Such information is necessary before a mechanistic role for hypomethylation of DNA in neoplastic transformation resulting from methyl deprivation can be proposed.

References

Boehm, T. L. J. & Drahovsky, D. (1983) *J. Natl. Cancer Inst.* **71**, 429–433

Chan, J. Y. H., Ruchirawat, M., Lapeyre, J.-N. & Becker, F. F. (1983) *Carcinogenesis* **4**, 1097–1100

Chiang, P. K. & Cantoni, G. L. (1979) *Biochem. Pharmacol.* **28**, 1897–1900

Cox, R. (1979) *Biochem. Biophys. Res. Commun.* **86**, 594–598

Cox, R. (1983) *Proc. Am. Assoc. Cancer Res.* **24**, 67

Cox, R., Prescott, C. & Irving, C. C. (1977) *Biochim. Biophys. Acta* **474**, 493–499

Ehrlich, M. & Wang, R. Y.-H. (1981) *Science* **212**, 1350–1357

Felsenfeld, G. & McGhee, J. D. (1982) *Nature (London)* **296**, 602–603

Gama-Sosa, M. A., Midgett, R. A., Slagel, V. A., Githens, S., Kuo, K. C., Gehrke, C. W. & Ehrlich, M. (1983) *Biochim. Biophys. Acta* **740**, 212–219

Harland, R. M. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2322–2327

Hoffman, D. R., Marion, D. W., Cornatzer, W. E. & Duerre, J. A. (1980) *J. Biol. Chem.* **255**, 10822–10827

Hoover, K. (1983) *Proc. Am. Assoc. Cancer Res.* **24**, 98

Hyde, C. L. & Poirier, L. A. (1982) *Carcinogenesis* **3**, 309–312

Kerr, S. J. & Heady, J. E. (1971) *Adv. Enzyme Regul.* **12**, 103–117

Kuo, K. C., McCune, R. A., Gehrke, C. W., Miggett, R. & Ehrlich, M. (1980) *Nucleic Acids Res.* **8**, 4763–4773

Linnell, J. C., Wilson, M. J., Mikol, Y. B. & Poirier, L. A. (1983) *J. Nutr.* **113**, 124–130

Marmur, J. (1961) *J. Mol. Biol.* **3**, 208–218

Mikol, Y. B. & Poirier, L. A. (1981) *Cancer Lett.* **13**, 195–201

Mikol, Y. B., Hoover, K., Creasia, D. & Poirier, L. A. (1983) *Carcinogenesis* **4**, 1619–1629

Newberne, P. M., Rogers, A. E., Bailey, C. & Young, V. R. (1969) *Cancer Res.* **29**, 230–235

Razin, A. & Riggs, A. D. (1980) *Science* **210**, 604–610

Sheid, B., Srinivasan, P. R. & Borek, E. (1968) *Biochemistry* **7**, 280–285

Shinozuka, H. & Lombardi, B. (1980) *Cancer Res.* **40**, 3846–3849

Shinozuka, H., Lombardi, B., Sell, S. & Iammarion, R. M. (1978) *Cancer Res.* **38**, 1092–1098

Shivapurkar, N. & Poirier, L. A. (1983) *Carcinogenesis* **4**, 1051–1057

Stein, R., Gruenbaum, Y., Pollack, Y., Razin, A. & Cedar, H. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 61–65

Wigler, M., Levy, D. & Perucho, M. (1981) *Cell* **24**, 33–40