Effects of 5-azacytidine and methyl-group deficiency on NAD(P)H:quinone oxidoreductase and glutathione *S*-transferase in liver

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Treatment with 5-azacytidine or dietary methyl-group deficiency effected DNA hypomethylation in mouse liver. With these treatments, NAD(P)H:quinone oxidoreductase (EC 1.6.99.2) and some glutathione *S*-transferase (EC 2.5.1.18) activities were over-expressed, lactate dehydrogenase (EC 1.1.1.27) activity was unaffected and the level of cytochrome *P*-450 was decreased. The 5-azacytidine induction of NAD(P)H:quinone oxidoreductase was significantly suppressed by puromycin, suggesting that increased enzyme activity results from an elevated level of enzyme-protein synthesis. Regulation at the transcriptional level was revealed by a substantial increase in mRNA of NAD(P)H:quinone oxidoreductase, as shown by Northern-blot analysis. The enzyme pattern observed with 5-azacytidine and with the (carcinogenic) dietary methyl-group deficiency resembles that found in hepatic nodules.

INTRODUCTION

DNA methylation and its functional significance in the control of gene expression is being intensively studied [1,2]. Methylation of specific cytosine residues in DNA is part of a multi-level control system in mammalian cells. It appears that hypomethylation can modulate gene expression, and evidence linking DNA hypomethylation to cancer is accumulating [3,4]. Methyl-group-deficient diets were found to be carcinogenic [5], with hepatic DNA being hypomethylated [6].

Gooderham & Mannering [7] observed that enzyme activities related to cytochrome *P*-450 were decreased by about one-third upon treatment of mice with 5-azacytidine, an agent known to lead to DNA hypomethylation. Here we examine the possible dependence of detoxication enzymes such as NAD(P)H:quinone oxidoreductase and GSTs on the extent of DNA methylation. The effects observed with 5-azacytidine were compared with those obtained with methyl-group-deficient diet. A preliminary account of the work with 5-azacytidine has been given [8].

MATERIALS AND METHODS

Chemicals

5-Azacytidine and the restriction enzymes *Eco*RI, *Hpa*II, *Msp*I and *Nco*I (Boehringer, Mannheim, Germany), agarose, ethidium bromide and puromycin (Sigma, München, Germany), CsCl (BRL, Neu-Isenberg, Germany), CDNB, DCNB, guanidine thiocyanate (Merck, Darmstadt, Germany), menadione (Fluka, Buchs, Switzerland), nitrocellulose filters (Schleicher und Schuell, Dassel, Germany), oligo(dT)-cellulose (Pharmacia, Freiburg, Germany) and deoxyadenosine

5'-[α -³²P]triphosphate (Amersham Buchler, Braunschweig, Germany) were obtained from the sources cited. All other chemicals were obtained from Boehringer, Sigma or Merck. The cDNA clone of NAD(P)H:quinone oxidoreductase, pDTD55, was generously given by Dr. C. Pickett (Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A.).

Animal pretreatment

Male NMRI mice (body wt. 25 ± 5 g) and male Sprague-Dawley rats (body wt. 240 ± 20 g) (from the Experimental Animal Department of this University) were held under standardized housing conditions with free access to standard stock diet (no. 1324) or special diets as indicated (all diets were from Altromin, Lage, Germany) and tap water.

5-Azacytidine. Groups of six to ten mice, or five rats, received one single dose of 25 mg of 5-azacytidine/kg body wt. by intraperitoneal injection under light diethyl ether anaesthesia and were killed 24 h later [7]. Controls received saline (0.9% NaCl) only. In an additional experiment, controls and 5-azacytidine-treated animals received puromycin (50 mg/kg) for 24 h.

Methyl-group deficiency. Mice were fed the methyl-group-deficient diet [no. 1070; DL-methionine (1.26 g/kg); choline chloride-free] over a period of 5 weeks, whereas the controls received the appropriate stock diet [no. C 1000; DL-methionine (8.64 g/kg); choline chloride (1.01 g/kg)]. At the end of pretreatment, animals were killed by cervical dislocation. The livers were quickly excised, washed in ice-cold saline, frozen in liquid N₂ and stored at -70°C .

Abbreviations used: t-BOOH, t-butyl hydroperoxide; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; GST, glutathione *S*-transferase; poly(A)⁺, polyadenylated.

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Table 1. Effect of 5-azacytidine on the activities of NAD(P)H:quinone oxidoreductase and GST isoenzymes in mouse liver

Enzyme activity of NAD(P)H:quinone oxidoreductase and GSTs was determined in the cytosolic fraction 24 h after the administration of 25 mg of 5-azacytidine and 50 mg of puromycin, as indicated, per kg body wt. Results are means $\pm$ S.E.M. for five to seven animals per group.

Enzyme	Activity (nmol/min per mg of protein)			
	Control	5-Azacytidine	5-Azacytidine + puromycin	Puromycin
NAD(P)H:quinone oxidoreductase	130 $\pm$ 15	216 $\pm$ 14	171 $\pm$ 12	178 $\pm$ 8
GST isoenzymes				
M I (t-BOOH)	694 $\pm$ 30	643 $\pm$ 46	—	—
M II (ethacrynic acid)	27.2 $\pm$ 3	40.5 $\pm$ 2	—	—
M III (DCNB)	66 $\pm$ 7	96 $\pm$ 7	—	—

Cell fractionation

Livers were homogenized in Tris/HCl (50 mmol/l) (pH 7.5)/KCl (25 mmol/l)/MgCl₂ (5 mmol/l)/EDTA-Na₂ (1 mmol/l)/sucrose (0.32 mol/l). Mouse liver nuclei were isolated from the 1000 g pellet by centrifugation in sucrose (2.4 mol/l) for 40 min at 124 000 g as described by Pfeifer *et al.* [9]. Cytosol and the microsomal fraction were obtained from the 9000 g supernatant after centrifugation at 105 000 g for 45 min.

Isolation of nuclear DNA

Genomic DNA was isolated from mouse liver nuclei by the Marmur [10] method as modified by Chandler *et al.* [11]. The purity and yield of the preparation were checked from the typical absorbances [12].

Determination of the extent of DNA methylation

H.p.l.c. method. Freeze-dried DNA was hydrolysed in 90 % formic acid (100 μ g/200 μ l) at 170 °C for 30 min. The sample was evaporated to dryness in a rotary evaporator, and the bases were dissolved in 50 % (v/v) acetic acid (100 μ g/100 μ l) as described by Eick *et al.* [13]. The bases were separated by cation-exchange h.p.l.c. (Merck-Hitachi, Darmstadt, Germany) on a Whatman Partisil 10 SCX column (0.46 cm $\times$ 25 cm) using isocratic elution with KH₂PO₄ (0.035 mol/l, pH 2.5), containing 5 % methanol at a flow rate of 2 ml/min [14]. Bases were detected by their *A*₂₇₀ and the extent of DNA methylation was calculated according to Singer *et al.* [15] and expressed as:

$$\text{Extent of methylation (\%)} = \frac{100 \times 5\text{-methylcytosine}}{\text{cytosine} + 5\text{-methylcytosine}}$$

Restriction-endonuclease-digest method. Isolation of high-*M*_r mouse liver DNA, restriction-enzyme digestion with the endonucleases *Msp*I and *Hpa*II and agarose-gel electrophoresis were performed essentially as described in [16]. The extent of DNA methylation was estimated from the densitometer scans (LKB 2202 Ultrosan laser densitometer) by the method of Kunnath & Locker [17].

Assays of cytochrome P-450 and cytosolic enzyme activities

Cytochrome P-450 was determined in the microsomal fraction as described by Omura & Sato [18]. Cytosolic

enzyme activities were generally determined at 25 °C as follows: NAD(P)H:quinone oxidoreductase with menadione as substrate [19]; GST with the substrates CDNB, DCNB, ethacrynic acid and t-BOOH [20]; and lactate dehydrogenase [21]. Protein was determined [22] with Precinorm S (Boehringer) as standard.

RNA isolation and Northern-blot hybridization

Total RNA was isolated from rat liver by the guanidine thiocyanate/CsCl procedure as described by Chirgwin *et al.* [23]. Poly(A)⁺ RNA was enriched by affinity chromatography on oligo(dT)-cellulose columns [24]. Approx. 5–10 μ g of poly(A)⁺ RNA were electrophoresed on 1 %-(w/v)-agarose slab gels containing formaldehyde (0.66 mol/l) [24]. After ethidium bromide staining (0.5 μ g/ml), RNA was blotted overnight on to nitro-cellulose filters by the method of Southern [25]. RNA was hybridized for 36 h at 42 °C against ³²P-labelled NAD(P)H:quinone oxidoreductase cDNA essentially as described by Davis *et al.* [24]. A *Nco*I/*Eco*RI fragment from plasmid pDTD 55 (Williams *et al.* [26]) was nick-translated to a specific radioactivity of approx. 1.5 $\times$ 10⁸ c.p.m./ μ g by using deoxyadenosine 5'-[α -³²P]triphosphate (400 Ci/mmol) [27]. Bound radioactivity was revealed by autoradiography at -70 °C with intensifying screens.

RESULTS

DNA hypomethylation with 5-azacytidine treatment

A single intraperitoneal injection of 5-azacytidine at a dose of 25 mg/kg body wt. results in a decrease in the extent of nuclear DNA methylation from 3.63 ($\pm$ 0.16) % to 3.10 ($\pm$ 0.31) % (*n* = 6) methylated cytosine bases after 24 h as determined by h.p.l.c. Similarly, by using the restriction-endonuclease-digest method as outlined in the Materials and methods section, the average state of methylation was found to be 75–85 % after 5-azacytidine treatment as compared with untreated controls (results not shown).

Induction of NAD(P)H:quinone oxidoreductase and GSTs with 5-azacytidine

The specific activities of NAD(P)H:quinone oxidoreductase assayed with menadione and of GST isoenzymes M II and M III assayed with ethacrynic acid and DCNB respectively [28] are increased in mouse liver 24 h after 5-azacytidine treatment, but no effect is

Fig. 1. Effect of 5-azacytidine on mouse liver enzyme activities 24 h after a single intraperitoneal injection

NAD(P)H:quinone oxidoreductase, GST (DCNB as second substrate) and lactate dehydrogenase were determined in the 9000 *g* supernatant; cytochrome *P*-450 was determined in the microsomal fraction. Values are means $\pm$ S.E.M. ($n = 4$).

Fig. 2. Time course of the 5-azacytidine (25 mg/kg body weight) effect on mouse liver enzyme activities

Enzyme activities were measured in cytosol fractions instead of in the 9000 *g* supernatant. Values are means $\pm$ S.E.M. ($n = 4$).

observed on GST isoenzyme M I, assayed with *t*-BOOH (Table 1). There was no significant effect with CDNB, a universal substrate for isoenzymes M I–III (results not shown). When puromycin, an inhibitor of translation, is administered to mice together with 5-azacytidine, an

increase of NAD(P)H:quinone oxidoreductase activity is substantially prevented, as would be expected if the increase in activity were due to an increase in enzyme protein.

The dose-dependence of the increase in the activities of NAD(P)H:quinone oxidoreductase and GST (DCNB) is shown in Fig. 1, and the time course of the increase of NAD(P)H:quinone oxidoreductase for 24 h is given in Fig. 2. The cytosolic marker enzyme lactate dehydrogenase is also shown in Fig. 1 and shows no significant change in its specific activity upon 5-azacytidine treatment. However, the cytochrome *P*-450 content responds to 5-azacytidine treatment by a decrease to about two-thirds of the control value, in agreement with previous results [7].

DNA hypomethylation and induction of NAD(P)H:quinone oxidoreductase and GSTs with methyl-group deficiency

The pattern of hypomethylation of DNA and of increased enzyme activities of NAD(P)H:quinone oxidoreductase and GSTs was also examined with methyl-group deficiency 5 weeks after feeding a methionine/choline-deficient diet. The data obtained are shown in Table 2 and resemble those obtained with 5-azacytidine.

Northern-blot analysis of NAD(P)H:quinone oxidoreductase mRNA

An increase of NAD(P)H:quinone reductase activity was also found in the livers of Sprague–Dawley rats (2.3-fold) 24 h after 5-azacytidine treatment, whereas, again, lactate dehydrogenase activity was unaffected. The rat liver NAD(P)H:quinone oxidoreductase cDNA clone pDTD 55 was used to investigate the expression of this

Fig. 3. Northern-blot analysis of rat liver mRNA with the cDNA probe pDTD55

Equal amounts of poly(A)⁺-enriched RNA from the livers of control and 5-azacytidine-treated rats were electrophoresed on an agarose slab gel (left). RNA was transferred to nitrocellulose filters, hybridized against NAD(P)H:quinone oxidoreductase cDNA [28] and revealed after 2 days exposure at -70°C (right). 18 S and 28 S rRNA, which are still contained in the poly(A)⁺ RNA preparation, served as internal size standards.

particular gene at the transcriptional level. For this purpose, liver mRNA was analysed by Northern blotting (Fig. 3). A 25-fold increase of the NAD(P)H:quinone oxidoreductase mRNA level in rat liver was determined by laser scanning of the autoradiograph 24 h after a single injection of 5-azacytidine.

DISCUSSION

Effect of 5-azacytidine and methyl-group deficiency on the extent of DNA methylation

The decrease by 15% of nuclear DNA methylation with 5-azacytidine was determined in mouse liver by two independent methods, from the absorbances after h.p.l.c. separation of the bases and, secondly, from the densito-

metric scans of agarose gels of specific restriction-enzyme digests. A similar decrease of DNA methylation was determined by feeding a methyl-deficient diet (consisting of low methionine and no choline, as compared with the control diet) for 5 weeks. In agreement with our results from mouse liver, recent studies with choline-free diets showed significant hypomethylation in total rat liver DNA [6,29]. In contrast, no changes in DNA methylation in male C3H mice were detected [30], suggesting differences between mouse strains.

Induction of NAD(P)H:quinone oxidoreductase and GSTs

The experiments with 5-azacytidine treatment and with dietary methyl-group deficiency reveal that there is a decrease in the cytochrome *P*-450 level, confirming previous work [7]. Concomitantly, there is an increase in the capacity of NAD(P)H:quinone oxidoreductase and some GST activities. With 5-azacytidine treatment the activities of the GST isoenzymes M II (ethacrynic acid) and M III (DCNB) are increased, whereas no effect was observed on isoenzyme M I (t-BOOH), in agreement with different regulation of the isoenzymes as suggested in [28]. Interestingly, in preneoplastic foci developing during chemical carcinogenesis in rat liver, the GST isoenzyme 3-3 is increased and 7-7 is expressed *de novo* [31]; these isoenzymes are immunologically related to the 5-azacytidine-inducible mouse enzymes M III and M II respectively. Recently, Tan *et al.* [32] reported an increase in isoenzyme subunits 2 and 3, but not in 1 and 4, in soluble liver supernatant fractions from rats fed a methyl-group-deficient diet.

Northern-blot analysis of NAD(P)H:quinone oxidoreductase

After 5-azacytidine treatment the level of NAD(P)H:quinone oxidoreductase mRNA is substantially increased in liver cells (Fig. 3). This observation is consistent with results of Williams *et al.* [26], who found increased amounts of NAD(P)H:quinone oxidoreductase mRNA associated with elevated enzyme activity in the livers of 3-methylcholanthrene-treated rats and in hyperplastic nodules developing in hepatic chemical carcinogenesis.

There is a considerable induction of NAD(P)H:quinone reductase at the transcriptional level (25-fold),

Table 2. Extent of DNA methylation and activities of detoxication enzymes in mouse liver after feeding a methyl-group-deficient diet

At 5 weeks after feeding a methionine-deficient, choline-free, diet the extent of DNA methylation (expressed as % of cytosine bases methylated) was evaluated. The activities of the detoxication enzymes were determined in the cytosolic fraction as described in the Materials and methods section. Results are means $\pm$ S.E.M. for four to six animals per group. Significance (Student's *t* test): *P < 0.05.

Parameter	Control diet	Methyl-group deficient diet
Extent of DNA methylation (%)	3.73 $\pm$ 0.22	3.36 $\pm$ 0.31*
NAD(P)H:quinone oxidoreductase activity (nmol/min per mg of protein)	183 $\pm$ 17	507 $\pm$ 49
GST activity (nmol/min per mg of protein)		
M II (ethacrynic acid)	24.7 $\pm$ 3	42.3 $\pm$ 12
M III (DCNB)	65 $\pm$ 4	106 $\pm$ 4
Lactate dehydrogenase activity (nmol/min per mg of protein)	2770 $\pm$ 170	3130 $\pm$ 340
Cytochrome <i>P</i> -450 amount (nmol per mg of protein)	0.80 $\pm$ 0.02	0.56 $\pm$ 0.03

but only a moderate increase in enzyme activity (about 2-fold) after treatment with 5-azacytidine. This effect could result from the existence of different isoenzymes [33,34], one isoenzyme representing most of the measurable activity in normal cells, the other isoenzyme being inducible and exhibiting its activity after treatment, for example, with 3-methylcholanthrene or 5-azacytidine.

Co-ordinate changes in enzyme patterns

It is noteworthy that the observation of a co-ordinate change of enzyme pattern after chemical pretreatment resembles numerous previous observations in carcinoma tissue and in hepatic nodules [35]. Several models for the regulation of enzyme expression of functionally related enzymes have been proposed. For example, a mechanism for the induction which depends on the generation of a labile oxidation-reduction signal has been postulated [36]. According to Nebert & Gonzalez [37], the expression of the five enzymes NAD(P)H:quinone oxidoreductase, GST (CDNB), UDP-glucuronyltransferase and the cytochrome *P*-450 species *P*₁450 and *P*₂450 is co-ordinately regulated by the aromatic *Ah* locus as a so-called 'gene battery'. Reference to the latter model makes DNA methylation an attractive regulatory mechanism for the expression of enzyme patterns in tumours [4]. In fact, Williams *et al.* [26] have recently shown that the NAD(P)H:quinone oxidoreductase gene is hypomethylated in persistent hepatocyte nodules as compared with the gene in liver tissue surrounding the nodules or in normal liver. Currently it is not known whether the genes coding for the mouse liver GST isoenzymes are hypomethylated in tumour tissue as well. From our observations with 5-azacytidine and with dietary methyl-group deficiency, such hypomethylation might be postulated for isoenzymes M II and M III.

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