[CANCER RESEARCH 43, 972-975, March 1983]
0008-5472/83/0043-0000$02.00

Regulation of O^6-Methylguanine-DNA Methyltransferase Levels in Rat Liver and Kidney[1]

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ABSTRACT

It was found that rat kidney contains a protein similar to that previously described in rat liver which catalyzes the transfer of the methyl group from O^6-methylguanine in DNA to a protein-bound cysteine residue. The amount of the renal O^6-methylguanine-DNA methyltransferase was increased up to 2.5-fold during renal hypertrophy in response to unilateral nephrectomy or treatment with folic acid. These results indicate that the protein in kidney resembles that in rat liver which is known to be increased in response to a variety of hepatotoxins or to partial hepatectomy. The liver O^6-methylguanine-DNA methyltransferase was reduced by hypophysectomy or thyroidectomy and could be increased by treatment with growth hormone or thyroxine. The level in the liver was considerably lower than the adult value in 1-day-old rats and increased to adult values by 14 to 21 days. At no time was the amount in the neonatal rat liver higher than in the adult, indicating that liver cell proliferation alone is not obligatorily coupled with an elevated methyltransferase level. The high sensitivity of neonatal rats to liver carcinogenesis by dimethylnitrosamine may be related to the high rate of cell proliferation and the lower capacity to repair O^6-methylguanine.

INTRODUCTION

A number of carcinogenic methylating agents are known to produce O^6-methylguanine in DNA. This product may lead to initiation of neoplastic growth if it persists in the cell long enough for DNA replication to take place, but rapid removal occurs in some tissues (19, 22, 29). The removal is brought about by the transfer of the methyl group to a cysteine residue on a protein acceptor molecule regenerating guanine directly in the DNA. Evidence for this mechanism has been provided for extracts from *Escherichia coli* (8, 28), rodent liver (4, 20, 38), human liver (37), and other human cells (45, 46). The bacterial protein which catalyzes this transfer is also the final acceptor of the methyl group and is not regenerated (16). Therefore, there is a stoichiometric relationship between the number of molecules of this protein present and the number of lesions which can be repaired (5, 16). Although the mammalian enzyme has not yet been purified to homogeneity, the transferase and acceptor activities copurify over a more than 1000-fold purification (38), and the kinetics of the reaction is also compatible with a stoichiometric reaction in which regeneration of the acceptor is negligible under the *in vitro* assay conditions (4, 37, 38, 46). Therefore, this activity has been described as both a methyltransferase and as a methyl acceptor protein. The former nomenclature is adopted in the present paper.

The amount of MT[3] present in a cell may relate to its susceptibility to carcinogenesis after methylation, but at present little is known about the factors regulating the level of this activity. Comparisons of the MT present in crude tissue extracts from a number of rat tissues indicated that this was greater in rat liver than in other organs but that some tissues, particularly kidney, were also able to remove O^6-methylguanine from a DNA substrate *in vitro* (30, 31). Since the reaction has been characterized only from the liver samples, it was not known if the lower activity present in extracts from kidney and other tissues was due to a similar protein. The hepatic MT was increased in response to alkylating agents (17, 18, 22-24, 35, 42, 43). Such an increase may indicate that the liver parenchymal cells [to which the increase is confined (43)] show an adaptive response to minimize the consequences of alkylation similar to that occurring in *E. coli* (5, 41). However, a variety of other hepatotoxins which are not metabolized to alkylating agents also induce MT in rat liver (6, 7, 27, 35), and MT was increased 6- to 7-fold following partial hepatectomy (35, 36). These results raise the possibility that the increased MT in response to hepatotoxins including alkylating agents may be a consequence of regenerative growth following cell death. It was not known whether increased MT could be detected in other tissues following stimuli leading to increased growth or whether other factors leading to liver growth influenced the hepatic MT. Therefore, the present experiments were carried out to determine: (*a*) whether MT could be characterized from rat kidney and if it was altered in this organ in response to growth after unilateral nephrectomy (13) or injury by exposure to folic acid (15, 44); and (*b*) whether hepatic MT varied significantly during neonatal growth or after treatment with growth hormone or thyroxine.

MATERIALS AND METHODS

Materials. *N*-[^{3}H]Methyl-*N*-nitrosourea (1.6 Ci/mmol) was obtained from New England Nuclear, Boston, Mass. Bovine growth hormone was a gift from the pituitary hormone distribution program of the National Institute of Arthritis, Metabolism and Digestive Diseases, NIH, Bethesda, Md. Other biochemical reagents were obtained from the Sigma Chemical Co., St. Louis, Mo.

Animals. Sprague-Dawley rats obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass., were used in all experiments. They were maintained under a controlled 12-hr-light-12-hr-dark cycle and allowed free access to water and food. Hypophysectomized rats or thyroidectomized female rats weighing about 150 g were compared to control female rats of the same weight and were used approximately 4 weeks after operation. Growth hormone dissolved in 0.9% (w/v) NaCl solution adjusted to pH 9 was injected s.c. at a dose of 1 mg/kg. Thyroxine was given at a dose of 50 μg/kg by injection of a solution in

[1] This work was supported by Grants CA-18137 and 1P30 CA-18450 from the National Cancer Institute.

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Received August 18, 1982; accepted November 23, 1982.

[3] The abbreviation used is: MT, O^6-methlyguanine-DNA methyltransferase.

0.9% (w/v) NaCl solution. Unilateral nephrectomy was carried out under ether anesthesia on male rats weighing about 220 g. Control rats were subjected to surgery under the same conditions, but no tissue was removed. Male rats weighing 240 to 260 g were treated with folic acid by i.p. injection of a solution of 50 mg/ml in 0.3 M $NaHCO_3$ at a dose of 250 mg/kg.

Assay of MT reaction. Tissue extracts were prepared and assayed for MT as described previously (36) using calf thymus DNA methylated by reaction with [^{3}H]methylnitrosourea as a substrate. Assays were conducted under conditions in which the amount of O^6-methylguanine removed was proportional to the amount of protein added, and results were expressed as pmol of O^6-methylguanine removed by an amount of extract equivalent to 1 g, wet weight, of tissue. The treatments studied did not alter the yield of protein obtained in the tissue extracts and, therefore, the changes observed in the MT levels would be exactly the same if the results were expressed per mg of protein assayed.

The standard assay medium for the MT assay contained 66 mM Tris-HCl, pH 8.3, 1.3 mM dithiothreitol, 0.1 mM EDTA, 1.3 mg of calf thymus DNA, the ^{3}H-methylated DNA (containing 2.6 pmol of O^6-methylguanine), and up to 15 mg of protein in a total volume of 3 ml. After incubation for various times at 37°, the reaction was halted by the addition of 1 M perchloric acid such that the final concentration became 0.25 M. The pellet was collected by centrifugation at 10,000 × g for 10 min, and the DNA was hydrolyzed by heating in 0.75 ml of 0.1 N HCl at 70° for 30 min. The mixture was then centrifuged to remove the pellet, and the supernatant was saved. The pellet was suspended again in 0.75 ml of 0.1 N HCl at 70° for 30 min and, after centrifugation, the supernatant combined with the earlier supernatant. The alkylated purines present in this supernatant were separated by high-performance liquid chromatography on Partisil 10SCX cation exchange medium (Whatman, Inc., Clifton, N. J.) eluted with 20 mM ammonium formate buffer, pH 4, at a flow rate of 2 ml/min (38). In some experiments, instead of using high-performance liquid chromatography separation, the O^6-methylguanine present was determined by precipitation with a specific antiserum with high affinity for this methylated base (38, 43) as follows. Aliquots of 0.5 ml of the hydrolysate were neutralized by addition of 0.06 ml of 1 M Tris base; then, 0.05 ml of a solution of bovine serum albumin (160 mg/ml), 0.05 ml of normal rabbit IgG (6 mg/ml), and 0.1 ml of antibody solution (2 mg/ml) were added, and the mixture was incubated at room temperature for 2 hr. An equal volume of saturated ammonium sulfate (pH 7.0) was then added. After mixing and standing for a further 10 min at room temperature, the pellet was collected by centrifugation and washed with 0.3 ml of water followed by addition of 0.3 ml of saturated ammonium sulfate. The precipitate was then dissolved in 0.2 ml of 0.2 N NaOH and assayed for radioactivity in the presence of 8 ml of ACSII Liquid Scintillation Cocktail. The efficiency of counting for ^{3}H was 21%.

The renal MT activity was characterized by measuring both the production of labeled *S*-methylcysteine using the ^{3}H-methylated DNA substrate described above and the formation of labeled guanine from a synthetic DNA substrate containing O^6-methyl-[8-^{3}H]guanine. The latter substrate (8, 21) was a generous gift from Dr. S. Mitra, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn. 37830. These experiments were carried out as described by Pegg *et al.* (37) for human liver extracts. Kidney protein was extracted and fractionated as described for rat liver (35).

RESULTS

The MT levels of liver extracts from rats of 1 to 42 days of age are shown in Chart 1. The amount at 1 day of age was only 21% of the normal adult value and rose to adult levels by 14 days. At no time was the level in the developing liver greater than the adult value. The MT in the maternal liver was, however, significantly greater than in control adult rats of either sex and did not decline to control values until some time after the end

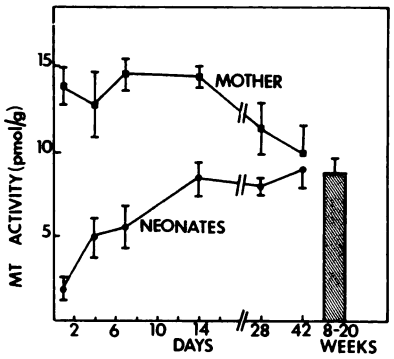

Chart 1. MT activity in rat liver during neonatal growth. Activities from rats of the ages indicated (●) and from the mothers of these rats (■) were measured. Results for MT activities are expressed as the mean for at least 5 estimations (young rats) or 4 estimations (mothers). The mean for adult (8 to 20 weeks) rats is also indicated (▨). *Bars*, S.E.

of the weaning period. It is likely that endocrine changes associated with pregnancy and nursing are responsible for this increase. Direct evidence for the endocrine regulation of MT in the liver is shown in Table 1. Hypophysectomy or thyroidectomy produced a significant decrease in the activity, and treatment with growth hormone or thyroxine increased it.

We have reported previously that O^6-methylguanine is lost from DNA after incubation with extracts prepared from rat kidney as well as rat liver, but this activity was not characterized (31). More detailed experiments have now shown that this activity is due to a methyltransferase similar to that found in liver. As shown in Table 2, the loss of O^6-methylguanine from DNA was accompanied by a stoichiometric formation of *S*-methylcysteine in protein and by the regeneration of guanine in the DNA substrate. The amount of the renal MT was increased both by unilateral nephrectomy and by treatment with folic acid (Chart 2). A maximal (2.5-fold) increase was produced 3 days after unilateral nephrectomy, and a 2-fold increase was seen at 2 days after exposure to folic acid. These increases peak at later times than does the increased DNA synthesis which occurs at 36 to 48 hr after unilateral nephrectomy (13) and at 24 to 48 hr exposure to folic acid (44).

DISCUSSION

The MT level is increased in adult rat liver during liver cell growth in response to partial hepatectomy (35, 36), toxic agents (6, 7, 22–24, 27, 35, 42), and hormones, but the present experiments indicate that there is no obligatory coupling between MT levels and the rate of growth. The amount was not increased during the period of early life in which the liver is actively growing. In fact, the MT shortly after birth was significantly lower than the adult value. This reduced level coupled with the high rate of cell division could contribute toward the sensitivity of newborn rats to liver carcinogenesis by single doses of dimethylnitrosamine (14). A possible contributory factor to the low neonatal MT could be the cellular composition of the liver at this time. We have shown recently that most of the hepatic MT resides in the hepatocytes, which have considerably higher levels than the nonparenchymal cells (43). The percentage of the liver mass made up by parenchymal cells is smaller during early stages of development. However, most of the hematopoietic cells present in the fetal liver are

Table 1

Endocrine effects on the activity of MT in rat liver

Treatment of hypophysectomized rats with growth hormone was for 6 days with doses of bovine growth hormone (1 mg/kg). Treatment with thyroxine consisted of 12 daily doses of 50 μg/kg. Both treatments resulted in significant increases in body weight. The MT activities were measured 12 hr after the final hormone injection. Results are shown for at least 6 animals.

Status of rats	MT activity (pmol/g, wet wt, liver)
Control unoperated	8.4 ± 0.8[a]
Hypophysectomized	4.2 ± 1.1
Hypophysectomized (treated with growth hormone)	8.7 ± 1.8
Thyroidectomized	4.3 ± 0.8
Thyroidectomized (treated with thyroxine)	7.6 ± 2.1

[a] Mean ± S.E.

Table 2

Properties of MT from rat kidney

Protein isolated from rat kidney was incubated for 60 min with DNA substrates, and the loss of O^6-methylguanine, formation of *S*-methylcysteine, and regeneration of guanine in the DNA was determined.

Amount of protein added (mg)	O^6-Methylguanine removed (pmol)	*S*-Methylcysteine formed (pmol)	Guanine formed in DNA (pmol)
1.8	0.32	0.26	0.34
3.6	0.68	0.62	0.66
5.4	0.91	0.89	0.95

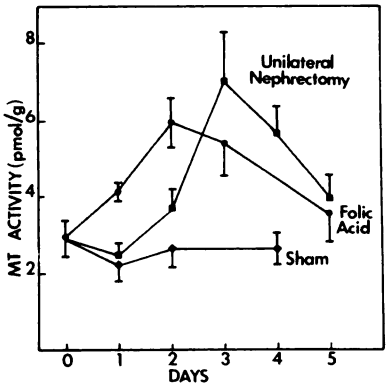

Chart 2. Effect of folic acid treatment and unilateral nephrectomy on renal O^6-methylguanine-DNA methyltransferase activity. Rats were treated with doses of folic acid (250 mg/kg) (●) or subjected to unilateral nephrectomy (■) or to sham operations (◆), and the MT activity was determined as indicated. Results are shown for 4 to 6 estimations. *Bars*, S.E.

lost by 1 day of age, and the volume of the liver taken up by parenchymal cells increases only slightly from Day 1 to Day 28 (10).

The endocrine-related changes in MT observed in the present paper are in agreement with earlier studies in which the persistence of O^6-methylguanine in liver DNA was studied *in vivo* after treatment with dimethylnitrosamine. The loss of O^6-methylguanine was found to be reduced upon hypophysectomy (34) or thyroidectomy (32) and was increased by thyroxin or growth hormone (32, 34). The present results indicate that these changes are brought about by alterations in the amount of MT. It is possible that increases in the MT after hepatotoxins or partial hepatectomy could also be mediated via alterations in hormone levels. It is not entirely clear how the endocrine status of rats affects carcinogenesis by alkylating agents. No effect of hypophysectomy on liver tumor incidence following dimethylnitrosamine was observed by Goodall (9). However, the dose of carcinogen used produced virtually 100% incidence of tumors in the unoperated group, and any enhanced sensitivity of the hypophysectomized rats may not have been detected. Hypophysectomized rats are thyroxine deficient owing to the absence of thyrotropin, and a significant enhancement of the carcinogenic action of dimethylnitrosamine towards both liver and kidney was seen in later experiments using thyroidectomized rats (25). In any case, the rate of cell proliferation in the liver may also be affected by endocrine oblation and may compensate for the reduced rate of removal of O^6-methylguanine.

Although all former studies demonstrating enhanced MT in response to treatment of animals with exogenous agents have been confined to rat liver (6, 7, 17–19, 22–24, 27, 35, 36, 42), the present experiments show clearly that rat kidney contains a similar system which is also inducible. Previous failures to enhance the rate of repair of O^6-methylguanine in the rat kidney by chronic treatment with dimethylnitrosamine (22, 23, 27) may be due to the very limited degree of interaction with the kidney and subsequent cell damage by the relatively low doses of the carcinogen used. The lesser MT in the rat kidney compared to liver correlates with the greater sensitivity of the kidney to tumor induction by single doses of dimethylnitrosamine (19, 22, 29). However, no direct information is at present available on the distribution of MT within the different cell populations making up the kidney, and the average value obtained in the present experiments may mask substantial differences between cell types. The treatment with folic acid used in the experiment depicted in Chart 2 induces a substantial proliferative response in the renal tubular cells (5, 44), and it appears likely that these cells, therefore, contain a significant proprotion of the MT.

The maximally enhanced MT level found in rat kidney occurs at a somewhat later time than the peak of DNA synthesis. This also resembles the situation in rat liver after partial hepatectomy (36) and does not support the argument that the increase in this DNA repair protein is designed to remove promutagenic lesions from DNA prior to cell division. There is good experimental evidence for such an induction in the case of uracil-DNA glycosylase, but in this case the increase occurs at an earlier time in the cell cycle (11, 12).

Attempts to demonstrate induction of MT in rodents other than rats have so far been unsuccessful (26). It is not clear whether rats are unique in this respect or whether appropriate experimental conditions for the other species have not yet been obtained. Very recently, Waldstein and Cao (45) observed an enhanced MT activity in HeLa cells exposed to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, suggesting that the induction is a more general phenomenon and may have similarities to the adaptive response in bacteria (5, 41). Also, Barrows and Shank (2), Becker *et al.* (3), and Quintero-Ruiz *et al.* (39) have demonstrated that O^6-methylguanine can be found in DNA from animals exposed to hepatotoxins which are not themselves alkylating agents. Endogenous methylation of purines in DNA may occur as a chemical reaction due to the reactivity of *S*-adenosylmethionine, a normal cellular component (1, 33, 40). It is conceivable that minor enzymatically mediated methylations of DNA may occur and play a role in cellular differentiation and gene expression. Any or all of these possibilities could require the MT to remove O^6-methylguanine from DNA, and this protein could play a role in normal cellular metabolism even in the absence of exogenous alkylation. The apparently paradoxical induction of this activity in response to stimuli which are not methylating agents could be due to their enhancement

of intracellular methylation. At present, MT can be assayed only by measuring the reaction with alkylated DNA. This indicates the amount of active protein present within the cell, but it is possible that the protein which has been utilized and contains *S*-methylcysteine at the acceptor site is also present. This would not be detected in these assays. Changes in the amount of assayable activity could be brought about by altered utilization of the protein for removal of endogenous methylation products. The development of specific antibodies to the MT protein which could be used to examine the amount of protein present irrespective of its methylation status would be of considerable interest.

ACKNOWLEDGMENTS

We thank Bonnie Merlino for manuscript preparation.

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