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Circadian variation in O^6-methylguanine-DNA methyltransferase activity in mouse liver

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Bifunctional chloroethylating cytostatic agents produce lethal DNA lesions, as a result of the formation of O^6-alkylguanines. These lesions can be repaired by O^6-methylguanine-DNA methyltransferase (MGMT). This ubiquitous nuclear and cytosolic enzyme removes the alkyl group by accepting it to the cysteine residue of its active site, thus preventing the formation of DNA interstrand cross-links. The role of the circadian organization in cellular protection against such DNA insults was examined in male B6D2F1 mice, synchronized with an alternation of 12 h of light and 12 h of darkness (LD12:12). MGMT activity was determined in liver of mice obtained at eight different circadian times, located 3 h apart. MGMT activity varied 5-fold along the 24 h time-scale, from 7 ± 1 pmol/g of tissue at 7 h after light onset (HALO), during the rest span, up to 32 ± 9 pmol/g at 19 HALO (second mid to late activity span). This large amplitude circadian rhythm in MGMT activity may be an important determinant of the susceptibility rhythms to alkylating agents. The greatest DNA repair activity occured at night when mice were active, eat and drink, and thus are at a higher risk of being exposed to chemical insults.

Key words: Chloroethylnitrosoureas, circadian, liver, mice, O^6-methylguanine-DNA methyltransferase, resistance.

Introduction

Circadian rhythms are genetically based and endogenous, since they persist in a constant environment.[1,2] The alternation of 12 h of light and 12 h of darkness (LD 12:12) for 3 weeks standardizes these rhythms in mice. As a result, peak and trough times of biologic functions become predictable, with reference to external time cues, such as light onset. Their time localizations have thus been expressed in hours after light onset (HALO). Mice are mostly active during the dark span (from 12 to 24 HALO) and rest during the light span (from 0 to 12 HALO). As a result of this circadian organization, the toxicity of many substances may vary largely and predictably according to time of exposure, both in laboratory rodents and in man. This applies in particular to anticancer drugs.[3–6] In mice, cystemustine, a new nitrosourea, was 20-fold less toxic following dosing in the mid to late activity span, as opposed to the rest of the span.[7]

It is well known that alkylating agents, such as chloroethylnitrosoureas (CENUs), interact with several of the DNA bases and at several sites of these bases, resulting in the formation of a variety of alkylated DNA lesions.[8] CENUs metabolism leads to the formation of a chloroethyl cation able to react on several nucleophilic sites of the nucleotide, the main cytotoxic lesion being O^6-chloroethylguanine, which is able to form covalent cross-links between complementary DNA strands.[9] O^6-alkylguanine repair is achieved by transfer of the alkyl group to an internal cysteine residue of the O^6-methylguanine-DNA methyltransferase (MGMT).[10–14] This reaction is stoichiometric, forming S-methyl cysteine in the protein and guanine in the DNA substrate. No other protein is involved in this transfer. In brief, the same protein acts as a methyltransferase and as a methyl acceptor protein.[15] The amount of this protein in the cells is inversely proportional to the DNA interstrand cross-link frequency and is consequently related to the cell sensitivity to CENUs. These mechanisms explain that the cytotoxicity of nitrosoureas may be counterbalanced by an increase in DNA repair activity within tumor cell,[13,16–18] and may result in a resistance to treatment.

Alkyltransferase activity has been detected in a wide variety of vertebrate species. The basic level of MGMT in mammals varies considerably from species

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to species,[11,12,19–22] but also from tissue to tissue within the same animal strain. The liver and the spleen contain relatively high levels even though brain and mammary gland present a slight activity.[15] Considerable differences in MGMT activity have been found in neoplastic tissues; cells exhibiting high levels of MGMT have been designated as Mer^+ and usually display resistance to nitrosoureas. MGMT-deficient cells were called Mer^-.[23–27]

Pre-treatment with DNA-methylating agents, such as *N*-methyl-*N*-nitrosourea (MNU) or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, inhibited the ability of Mer^+ cells to dealkylate chloroethyl monoadducts by saturation of their repair system.[28] This finding suggested that the persistence of monoadducts, the formation of interstrand cross-links or both events were toxic to the cells.

Circadian changes in fluoropyrimidine toxicities were shown to be related to rhythms in cellular activities of enzymes involved in drug catabolism (dehydropyrimidine dehydrogenase) or in the anabolism of their cytotoxic forms (uridine phosphorylase, orotate phosphoribosyl transferase and thymidine kinase).[29–31] We have examined whether the activity of MGMT, a DNA repair enzyme, also varied along the 24 h time-scale in mice. MGMT activity level was assessed in the liver because this organ possesses the highest activity in mice B6D2F1 (Table 1) and because liver allows us to easily obtain sufficient amounts of material, so as to realize repeated assessments. This rhythm might constitute one of the mechanisms of susceptibility rhythms to DNA alkylating agents.

Materials and methods

Animals and synchronization

Mice were housed two per cage with free access to food and water. They had been synchronized, in a chronobiologic animal facility (ESI Flufrance, Arcueil, France), for 3 weeks before study in order to adjust their biologic rhythms. The lighting regimen consisted of an alternation of 12 h of light (L) and 12 h of darkness (D) (LD12:12). This facility had six soundproof, temperature-controlled (23 ± 1°C) compartments provided with filtered air (120 ± 10 l/min), each compartment having its own programmable lighting regimen. Fourty-one male B6D2F1 mice were randomly allocated to one of eight groups upon arrival (IFFA-CREDO, L'Arbresle, France), each group corresponding to a different circadian stage of study. Mice were 9–10 weeks old at the end of the standardization procedure.

Adequate synchronization of mice was documented by the observation of a normal circadian rhythm in rectal temperature of all mice.

Table 1. MGMT activity (± SEM) in normal tissues of B6D2F1 mice[32]

Tissue	*N*	MGMT (fmol/mg protein)
Liver	4	82 ± 21.5
Spleen	2	77 ± 8.5
Lung	2	19 ± 1.4
Kidney	2	23 ± 1.4
Brain	2	10.5 ± 0.7

MGMT determination

After synchronization, mice were sacrified, then bled. Liver was removed, weighed, frozen in liquid nitrogen and kept at −80°C until MGMT determination. MGMT assay was performed in five or six mice obtained at eight different circadian stages (1, 3, 7, 11, 13, 15, 19 and 23 HALO).

The assessment technique was radiochemical, using tritium. The substrate, O^6-[^{3}H]methylguanine-DNA, was obtained by methylation of deproteinized DNA. O^6-[^{3}H]methylguanine-DNA is a highly specific substrate of MGMT, similar to the endogenous substrate of this enzyme. Its use allowed an adequate estimation of alkyltransferase activity in cellular extracts.[33] DNA methylation with [^{3}H]MNU led to methylphosphotriester formation, with methylated purines and pyrimidines.[34,35] Main alkylations occurred on the N^7 and O^6 position of guanines, and N^3 of adenines. However, the quality of DNA methylation by [^{3}H]MNU was not fully reproducible, with somewhat different yields. The three main tritiated bases were separated by HPLC, after acid hydrolysis of the substrate.[35] Following protein incubation with substrate, the radioactivity transferred from O^6-[^{3}H]methylguanine on protein was proportional to the alkyltransferase activity. The difference between the initial amount of O^6-[^{3}H]methylguanine and that which remained after the reaction corresponded to the amount of methyl transferred on protein.

Preparation of liver protein extract

A separate protein extract was prepared with each liver. Liver homogenate was obtained after thawing

at 4°C, addition of 4 volumes of lysis buffer (70 mM HEPES, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol, 400 mM NaCl, 10% glycerol, 1 μg/ml leupeptine–antipain–aprotinine) and attrition by Ultraturr at medium speed. This suspension was centrifuged for 15 min at 12 000 *g*. Supernatant was kept at 4°C, but pellet was resuspended in 2 ml lysis buffer and sonicated three times for 45 s each at 1 min intervals, before a last 30 min centrifugation at 40 000 *g*. Both supernatants were mixed and proteins assessed by the Coomassie blue method. The protein extract obtained (about 5 ml/liver) was aliquoted and frozen at −80°C, then at −196°C.

Preparation of substrate

The substrate for the assay consisted of calf thymus DNA (Sigma, St Quentin Fallavier, France) which had been methylated by reaction with [^{3}H]MNU (Amersham, 592 GBq/mmol or 16 Ci/mmol) as previously described.[35] Under these conditions, methylated DNA contained 1 ± 0.3 fmol of O^6-methylguanine and 8.5 ± 1 fmol of N^7-methylguanine per μg of methylated DNA. Substrate specific activity was 220 c.p.m./μg of methylated DNA. O^6-methylguanine and N^7-methylguanine accounted, respectively, for 6 and 50% of the total radioactivity.

MGMT assay

MGMT assay was carried out on liver protein extracts. These were incubated at 37°C for 30 min with 80 μg of methylated DNA in the incubation buffer (70 mM HEPES, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol) for a total volume of 140 μl. Samples were then cooled at 0°C. After addition of 25 μl of 2N HCl, samples were heated at 80°C for 30 min, then neutralized by 2N KOH; then O^6-methylguanine (Chemsyn Science Laboratories, Lenexa, KN) and N^7-methylguanine (Sigma) (50 mmol/10 μl) were added as internal HPLC standards (final volume = 200 μl). Methylated bases were separated by reverse-phase HPLC using a C_{18} Kromasil 10 μm, 30 cm, column eluted at 2 ml/min by a 20 mM $NH_4H_2PO_4$ (pH 5.0) mobile phase containing 10% (time 0–10 min), 15% (time 10–30 min) or 10% (time 30–35 min) methanol. The retention times were 11 min for N^7-methylguanine and 22 min for O^6-methylguanine. Successive 2 ml fractions were collected with an automatic fraction collector and counted in a Packard Tricarb 4530 scintillation spectrometer. Methyltransferase activity was determined by measuring the disappearance of O^6-methylguanine from the substrate. Each liver extract was assessed three times with three different protein concentrations. Mean MGMT activity was calculated for each mouse liver.

Statistical analyses

Mean and SEM were calculated for each variable. The statistical significance of differences observed between groups was validated with non-parametric analysis of variance (Kruskall–Wallis ANOVA).

Data were also analyzed for circadian rhythmicity (with a period, τ, defined as ~24 h) by the cosinor method.[36] A rhythm was characterized by three parameters: the mesor (24 h adjusted mean), the double amplitude (difference between the minimum and the maximum of the fitted cosine function) and acrophase (∅, time of maximum, with light onset as ∅ reference). Mesor, double amplitude and ∅ were obtained with their 95% confidence limits if a rhythm was detected. This was achieved when the amplitude A differed from zero (non-null amplitude F-test) with $p < 0.05$. The significance of both conventional and chronobiologic statistics was needed for validating temporal changes as rhythms.[36]

Results

Synchronization of mice

A circadian rhythm in rectal temperature was statistically validated by the cosinor method for each study on the day of sampling ($p < 0.001$). Temperature acrophase was localized in the second half of the dark activity as it is usually the case (20^{15} HALO ± 89 min), thus establishing an adequate synchronization of mice.

MGMT determination

Liver weight varied as a function of hour of sacrifice, from 1.144 ± 0.003 g at 15 HALO to 1.513 ± 0.003 g at 7 HALO. Cosinor analysis confirmed a circadian rhythm with an acrophase at 2^{50} HALO ± 67 min ($p < 10^{-5}$).

MGMT activity displayed an even more pronounced circadian rhythm. Mean value (± SEM) varied 9-fold, from 38.8 ± 5.7 fmol/mg protein at 7 HALO up to 363.6 ± 81.8 fmol/mg protein at 19

Table 2. Mean values (± SEM) of MGMT activity, protein content and liver weight in B6D2F1 mice, according to sampling time

HALO	*N*	MGMT (fmol/mg protein)	MGMT (pmol/g liver)	MGMT (pmol/liver)	Proteins (mg/g liver)	Proteins (mg/liver)	Liver weight (g)
1	5	145.0 ± 15.8	16.42 ± 2.23	24.36 ± 2.06	112.6 ± 10.1	167.0 ± 7.3	1.496 ± 0.130
3	5	169.2 ± 28.4	24.78 ± 2.59	36.72 ± 4.36	148.2 ± 14.1	218.9 ± 17.7	1.480 ± 0.006
7	6	*38.8 ± 5.7*	*6.98 ± 0.98*	*10.56 ± 1.28*	181.5 ± 23.5	**273.8 ± 30.1**	**1.513 ± 0.003**
11	5	64.4 ± 6.9	12.78 ± 2.73	15.02 ± 2.25	171.0 ± 19.8	202.0 ± 17.8	1.188 ± 0.008
13	5	172.2 ± 50.4	32.12 ± 9.49	34.66 ± 9.98	**186.4 ± 5.1**	201.6 ± 4.6	*1.082 ± 0.012*
15	5	153.6 ± 65.5	22.46 ± 9.40	25.72 ± 10.79	145.4 ± 4.4	166.7 ± 7.3	1.144 ± 0.003
19	5	**363.6 ± 81.8**	**32.02 ± 8.80**	**41.68 ± 9.29**	87.2 ± 3.8	114.6 ± 3.7	1.317 ± 0.008
23	5	130.0 ± 19.0	10.56 ± 1.63	14.66 ± 1.85	*81.2 ± 5.9*	*112.8 ± 3.1*	1.400 ± 0.123
p[a]	41	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Values at peak and trough times are given in italic and bold type, respectively.
[a] *H* test (Kruskall–Wallis).

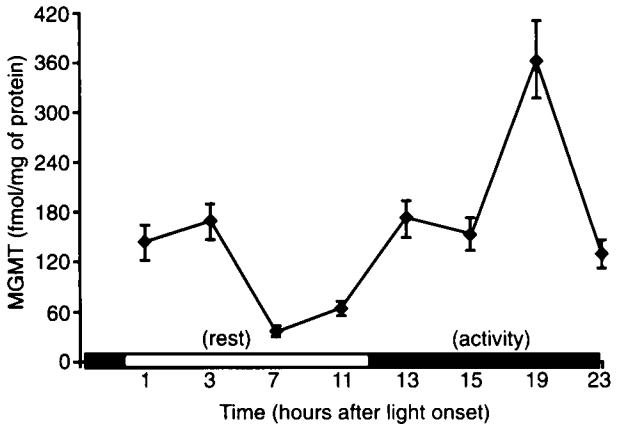

Figure 1. Liver MGMT activity (fmol/mg protein) according to circadian sampling time in male B6D2F1 mice. Mean values ± 1 SEM differed with statistical significance ($p < 0.001$ from Kruskall–Wallis ANOVA). Cosinor analysis showed an acrophase occuring at 19^{17} HALO ± 77 min ($p < 10^{-5}$).

HALO (Table 2 and Figure 1). The circadian acrophase was estimated at 19.17 ± 77 min with cosinor analysis (p from ANOVA and cosinor < 0.001) (Table 3). Results were similar if MGMT activity was expressed per g of fresh tissue or per total liver.

Discussion

Circadian variations in enzymatic activities or other cellular functions markedly influence drug metabolism, with an even increased likehood if the drug half-life is short.

CENUs exert their toxicity mostly through the formation of O^6-chloroethylguanine, which, in turn, forms covalent cross-links between the complementary DNA strands.[9,37–39] The enzyme MGMT is involved in the early repair of these lesions. Thus, MGMT is able to protect cells from lethal effects of

Table 3. Circadian rhythms in MGMT activity, protein content and liver weight in B6D2F1 mice liver: results from cosinor analysis

Variable	p[a]	Mean (± SE)	Amplitude (± SD)[b]	Acrophase (HALO) (± SD)[c] (min)
Liver weight (g)	10^{-4}	1.3 ± 0.02	0.20 ± 0.05	2^{50} ± 67
Proteins (mg/g liver)	10^{-5}	139.1 ± 2.7	55.3 ± 10.6	9^{10} ± 42
MGMT activity (fmol/mg protein)	10^{-5}	154.6 ± 11.4	116.9 ± 46.4	19^{17} ± 77
MGMT activity (pmol/g liver)	0.003	19.6 ± 1.5	8.5 ± 6.0	17^{34} ± 143
MGMT activity (pmol/liver)	0.017	26.0 ± 1.8	8.9 ± 7.5	18^{55} ± 204

[a] From an *F* test of rejection of the null amplitude hypothesis.
[b] Difference between maximum and minimum of the cosine function.[36]
[c] Time of maximum in cosine function.

exposure to chloroethylating agents such as BCNU, CCNU, fotemustine, etc. The capacity to repair O^6-alkylguanine formed in DNA is related to both the content of the repair enzyme at the time of drug exposure and the rate at which this enzyme can be synthesized to replace that used up in the repair reaction.[15]

Indeed, liver MGMT activity displayed a 9-fold variation along the circadian cycle, being lowest at 7 HALO and highest at 19 HALO. These results were in good agreement with the changing cytotoxicity of nitrosoureas according to cellular MER phenotype. They stressed that such phenotype may exhibit a large physiologic variation in its expression along the 24 h time-scale in normal cells.

A 50–70% change in liver weight has long been known in mice or rats along the 24 h time-scale. This rhythm was partly related to nocturnal food intake.[40,41] However, the large difference in the respective amplitudes of the circadian variations in liver weight and MGMT, as observed in the present study, supports the fact that the rhythm in MGMT activity was no more than a reflection of liver weight.

Circadian rhythms have been shown for several enzymatic activities involved in the cytotoxicity of anticancer compounds. Dehydropyrimidine dehydrogenase (DPD) (an enzyme which catabolizes fluoropyrimidines), orotate phosphoribosyl transferase (OPRT), uridine phosphorylase and thymidine kinase (TK) all displayed 2- to 8-fold changes in their activities in normal cells of mice (Table 4).[29–31,42–43] Moreover, the rhythms in DPD and TK activities were synchronous in liver, in intestinal mucosa, in spleen and in bone marrow.

The cellular enzymatic activities which catabolize or anabolize cytotoxic compounds may vary, with more than a doubling potential along the circadian cycle. Reduced glutathione, which protects cells against electrophilic reactive compounds, also presents with a similar chronobiologic variation potential.[44]

Conclusion

We offer here the first demonstration that MGMT, an enzyme involved in DNA repair, displayed a large amplitude circadian rhythm.

These results clearly point out the fact that biochemical and molecular events involved in cell survival are largely coordinated along the circadian time-scale.

Furthermore, inhibitors of MGMT activity, which are presently being developed for enhancing nitrosourea anticancer efficacy, may also benefit from selective circadian timing.

Table 4. Cellular mechanisms involved into the chronopharmacology of anticancer drugs

Biochemical variable	Tissue	Circadian variation coefficient	Time (HALO) of		Reference
			High	Low	
Protection					
[GSH]	liver, jejunum colon	2	18–24	8	Li *et al.* (submitted)
Catabolism					
dehydropyrimidine dehydrogenase	liver, bone marrow	2–3	3–7	15–19	29 30 31
Anabolism					
uridine phosphorylase	liver	4	15–18	3–6	30
orotate phosphoribosyl transferase	liver	2	15–18	3–6	30
thymidine kinase	liver, spleen; intestine, bone marrow	2–5	22	10	31
Repair					
O^6-alkylguanine-alkyltransferase	liver	9	19	7	Martineau *et al.* (present data)
Other					
thymidine phosphorylase	liver	0	—	—	42 30
dehydrofolate reductase	liver	2.5	18	0	43

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