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Induced Synthesis of O^6-Methylguanine-DNA Methyltransferase in Rat Hepatoma Cells Exposed to DNA-damaging Agents

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When the rat hepatoma cell line H4IIE was treated with DNA-damaging agents such as N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), ultraviolet light and γ-rays, the O^6-methylguanine-DNA methyltransferase activity increased 2 to 3 times over the level seen in non-treated cells. SDS/polyacrylamide gel electrophoresis followed by fluorography revealed that a single species of methyltransferase protein with a molecular weight of 25,500 was present in both non-treated and treated cells. Northern blot analysis using a cloned rat cDNA as a probe revealed that the enzyme activity increased because transcription of the gene was enhanced. The level of enzyme activity increased within 48 h after UV irradiation and remained at a higher level for 150 h. Following UV irradiation, the cells become more resistant than the normal cells to MNNG.

Key words: Alkylation — DNA repair — Induction

Various organisms possess O^6-methylguanine-DNA methyltransferase, which repairs alkylated bases in DNA. The enzyme transfers methyl groups from O^6-methylguanine and other methylated moieties of the DNA to the enzyme molecule. There is evidence to indicate that the enzyme plays important roles in cellular defence against mutagens and carcinogens with alkylating activity.[1-4]

cDNA for the human methyltransferase has been cloned and its structure elucidated.[5-7] Inspection of the predicted amino acid sequence revealed that the human enzyme resembles bacterial methyltransferases, Ogt protein of *Escherichia coli* and Dat protein of *Bacillus subtilis.*[8, 9] In addition to these constitutive enzymes, bacteria possess inducible enzymes.[10-12] The inducible methyltransferase of *E. coli,* Ada protein, carries two distinct functions, one to repair methylated moieties of the DNA and the other to act as a positive transcriptional regulator.[13-15] The amount of Ada protein molecules increases considerably when the cells are exposed to low concentrations of alkylating agents.

Mammalian tissues contain relatively high levels of methyltransferase activity[16] and it is sometimes an issue whether the activity increases after treatment with alkylating agents. Laval *et al.* [17, 18] reported that O^6-methylguanine-DNA methyltransferase activity in the rat hepatoma cell line H4 increased when the cells were exposed to various DNA-damaging agents, including UV and γ-rays. An increase in the enzyme activity was also observed with rat liver tissues.[19] Although these phenomena were initially regarded as a process analogous to adaptive response observed in *E. coli,*[20, 21] subsequent studies revealed that there are important differences between the two phenomena. The spectra of the inducing agents found in rat cells are divergent as compared with those for *E. coli,* where only alkylating agents are effective.[22] The increase in enzyme activity in rat cells is much less compared with the extent attained in *E. coli* cells. In addition, while observations have been made in rats, cells in other rodents revealed no such findings.[14-19]

We recently cloned a rat cDNA for O^6-methylguanine-DNA methyltransferase, using as a probe previously isolated human cDNA (K. Sakumi *et al.*, in press). This clone has facilitated examination of the levels of expression of the methyltransferase gene in rat cells exposed to various agents.

MATERIALS AND METHODS

Cells The H4IIE cell line, derived from rat hepatoma,[23] was kindly provided by Dr. Toshikazu Nakamura of Kyushu University. The cells were maintained in Dulbecco's modified Eagle's (DME) medium containing 10% fetal calf serum, 100 μg/ml streptomycin and 100 units/ml penicillin G in a humidified 95% air-5% CO_2 at 37°C. Cells in the exponential phase of growth were harvested for experiments.

Chemicals MNNG was purchased from Tokyo Kasei Co. (Tokyo). [^{3}H]MNU (0.5 Ci/mmol and 17.7 Ci/mmol) and [α-^{32}P]dCTP (3,000 Ci/mmol) were obtained from Amersham.

Treatment of cells For MNNG treatment, the cultured cells were washed with serum-free DME medium (pH

The abbreviations used are: MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; UV, ultraviolet light; MNU, methylnitrosourea; DME, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate.

6.0) and incubated at 37°C for 1 h in the same medium containing various concentrations of MNNG. The medium containing this agent was removed and the cells were incubated in a fresh medium. For UV and γ-ray irradiation, the medium was removed and cells were exposed to various doses of irradiation. UV irradiation was performed with a germicidal lamp (Toshiba) at a distance of 60 cm and at a rate of 0.45 $J/m^2/s$. A ^{137}Cs γ-ray irradiator (Gammacell 40, Atomic Energy of Canada, Ottawa) was used for γ irradiation at a rate of 1.25 Gy/min.

Assay of methyltransferase activity The enzyme activity was measured as described by Hayakawa *et al.*[5] The cells were harvested by low-speed centrifugation, frozen and stored at −80°C until use. The cells (1×10^7) were resuspended in 1 ml of 50 m*M* Tris-HCl (pH 7.8)-0.1 m*M* EDTA-1 m*M* DTT-1 m*M* PMSF-70 m*M* NaCl, and sonicated. After centrifugation at 100,000*g* for 10 min at 4°C, the supernatant was taken as a crude extract and this extract was incubated at 37°C for 15 min in 70 m*M* Hepes-KOH (pH 7.8)-1 m*M* DTT-5 m*M* EDTA-1.6 μg/ml [^{3}H]MNU (0.5 Ci/mmol)-treated calf thymus DNA (23,600 dpm/μg) in a final volume of 100 μl. The reaction was terminated by adding 200 μl of 0.8 *M* TCA followed by heating at 90°C for 15 min. After centrifugation, the precipitate was collected and washed with 500 μl of 5% TCA. The acid-insoluble material was dissolved in 100 μl of 0.1 *M* NaOH and neutralized with 0.1 *M* HCl, and the radioactivity counted. To analyze proteins on SDS/polyacrylamide gels, the reaction was performed with 1.25 μg [^{3}H]MNU (17.7 Ci/mmol)-treated calf thymus DNA (196,000 dpm/μg) in a final volume of 60 μl, and stopped by addition of 0.33 volume of 0.25 *M* Tris-HCl (pH 6.8)-20% (v/v) mercaptoethanol-8.8% (w/v) SDS-40% glycerol, followed by heating at 95°C for 5 min. The mixtures were then electrophoresed on SDS/14% polyacrylamide gels, and ^{3}H-labeled proteins were detected by fluorography.[24]

Cell survivals Cells were seeded at concentrations of 400 to 1,000 cells/100-mm dish, incubated for 24 h, and then treated with MNNG or UV. After incubation for 10 to 14 days, the cells were fixed with 10% (v/v) formaldehyde and stained with 0.1% (w/v) crystal violet. The colonies were observed under a magnifying glass and counted.

RNA preparation and Northern blot analysis Total RNAs were extracted using the guanidium thiocyanate/CsCl method[25] and poly(A)$^+$mRNAs were purified by using Oligotex-dT30 (Takara, Kyoto). For Northern blot analysis, 3 μg samples of poly(A)$^+$mRNAs were electrophoresed on 1.5% agarose gels and blotted onto nylon Hybond-N$^+$ (Amersham). Membranes were prehybridized for 14 h at 42°C in 50% formamide-5$\times$SSPE-5$\times$Denhardt's solution-0.5% SDS-50 μg/ml heat-denatured salmon sperm DNA. Then ^{32}P-labeled cDNA for rat O^6-methylguanine-DNA methyltransferase was added and hybridization was performed for 36 h at 42°C. Membranes were washed at 65°C in 1$\times$SSC-0.1% SDS. The data were processed in a Fujix Bas2000 Bio-Image Analyzer.

RESULTS

Methyltransferase activity in cells treated with DNA-damaging agents H4IIE cells were treated with various DNA-damaging agents and levels of O^6-methylguanine-DNA methyltransferase activity in the cells were determined. The results obtained are summarized in Table I. After exposure of cells to MNNG, UV or γ-rays (survival rate 90 to 30%), the enzyme activity increased 2 to 3 times over the level in the untreated cells.

Fig. 1 shows quantitative measurements of the enzyme activity. Under the conditions used, the activities are proportional to the amounts of the extracts, and two to three times higher levels of the enzyme activity were attained with treated cells, as compared to that for non-treated cells.

The time-course of increase of the enzyme activity in cells that were UV-irradiated at 10 J/m^2 is shown is Fig. 2. The level of enzyme activity increased within 48 h after irradiation and then leveled off. The increased level of activity remained even 150 h after irradiation.

Table I. Methyltransferase Activity in H4IIE Cells Treated with Various DNA-damaging Agents

Treatment		Survival (%)	Methyltransferase activity (fold)	
			48 h	72 h
None (control)		100	1.0	ND[a]
MNNG	15 μM	97	1.5	1.7
	50 μM	28	2.2	2.9
UV	5 J/m^2	95	2.8	2.7
	10 J/m^2	54	2.3	2.2
γ-rays	3 Gy	ND	2.0	2.0
	5 Gy	ND	2.8	3.0

a) Not done.

For treatment with MNNG, H4IIE cells were washed with serum-free DME medium (pH 6.0) and then incubated at 37°C for 1 h in the same medium containing various concentrations of MNNG. After the treatment, the cells were washed and incubated in fresh medium. For irradiation with UV or γ-rays, the medium was removed and cells were exposed to various doses of irradiation, then incubated in fresh medium for 48 and 72 h and collected to prepare crude extracts. The methyltransferase activity in the non-treated cells was 2 pmol/15 min/mg protein, a value taken as the control (1.0).

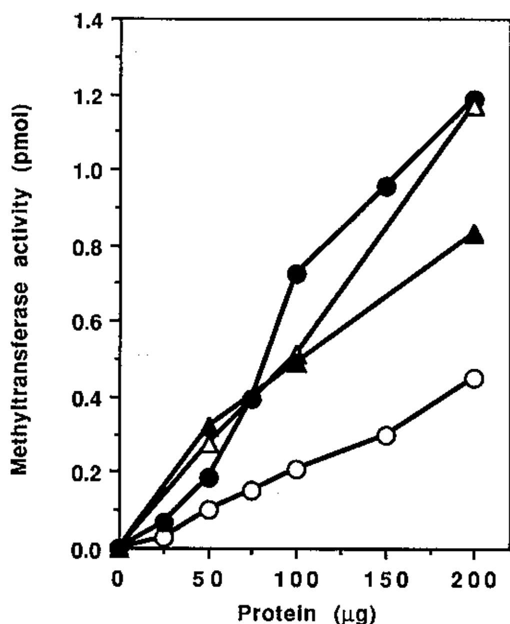

Fig. 1. Methyltransferase activity in H4IIE cells after various treatments. H4IIE cells were treated with 50 μM MNNG or irradiated with UV (5 J/m^2) or γ-rays (5 Gy), then incubated at 37°C for 48 h. The enzyme activity is expressed in terms of amount of methyl groups transferred from [^{3}H]MNU-treated DNA to the acid-insoluble fraction in 15 min. (○) non-treated (control) cells; (●) cells treated with MNNG; (△) UV-irradiated cells; (▲) γ-irradiated cells.

Electrophoretic analysis of the methyltransferase protein Since the methyltransferase protein accepts a methyl group from [^{3}H]MNU-treated DNA and retains it on the molecule, the nature of the induced activity can be analyzed by SDS/polyacrylamide gel electrophoresis followed by fluorography. Crude extracts were prepared from normal cells and cells exposed to various DNA-damaging agents and subjected to reactions with [^{3}H]-MNU-treated DNA. As shown in Fig. 3, a distinct band corresponding to a 25,500-dalton protein was detected. Densitometric determination revealed that samples of MNNG-treated, UV-irradiated and γ-irradiated cells contain 2.3, 2.2 and 2.3 times higher levels of radioactivity, respectively as compared with the non-treated sample. Although the fluorographic analysis showed an additional faint band at a position corresponding to a protein with a molecular weight of 26,000, it may represent a modified form of the protein.

Enhanced expression of the methyltransferase gene after UV irradiation Levels of expression of the gene after UV irradiation were examined using Northern blot analysis. H4IIE cells were irradiated at 5 and 10 J/m^2 and incubated for 48 h after irradiation. Poly(A)$^+$mRNAs were then extracted from the cells and hybridized with a rat cDNA. As shown in Fig. 4, more intense bands were detected in the irradiated samples than in the non-irradiated one; amounts of the transcripts in cells irradiated at 5 and 10 J/m^2 were 2.6- and 3.7-fold more

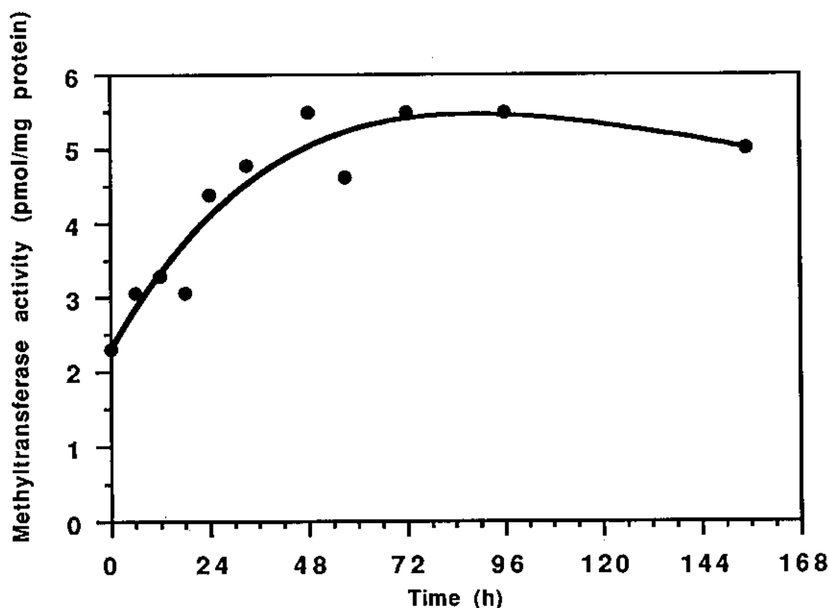

Fig. 2. Kinetics of induction of methyltransferase activity in H4IIE cells after UV irradiation. H4IIE cells were UV-irradiated at 10 J/m^2 and then incubated in fresh medium at 37°C for various periods of time. The enzyme activity was determined as described.

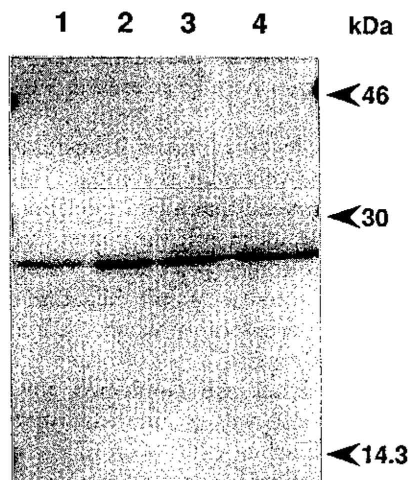

Fig. 3. Fluorography of the methyltransferase reaction products. Reaction mixtures containing 60 μg protein of crude extracts were subjected to electrophoresis on SDS/polyacrylamide gels. Lane 1, untreated cells; lane 2, cells treated with 50 μM MNNG for 1 h; lane 3, cells irradiated with UV at 5 J/m^2; lane 4, cells irradiated with γ-rays at 5 Gy.

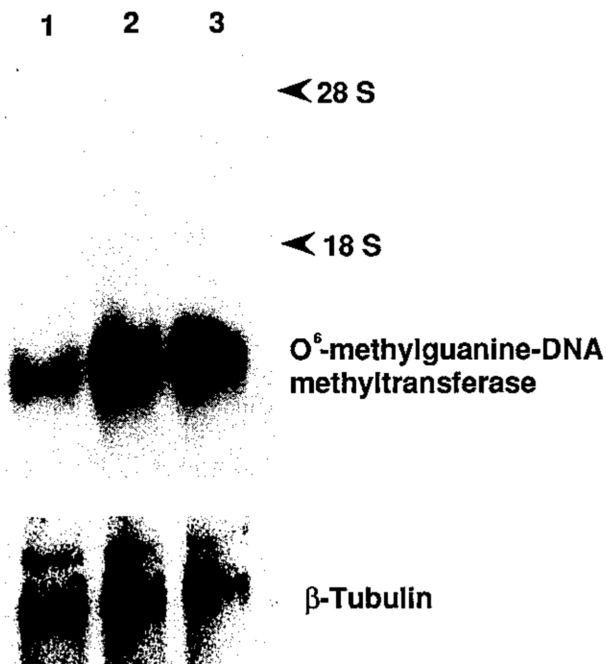

Fig. 4. Northern blot analysis of mRNAs from H4IIE cells irradiated with UV. mRNAs were prepared from H4IIE cells 48 h after UV irradiation. Poly(A)$^+$ mRNAs (3 μg each) were electrophoresed on 1.5% agarose gels, blotted and hybridized with cDNAs for rat O^6-methylguanine-DNA methyltransferase and for β-tubulin. The positions of ribosomal RNAs are indicated with arrowheads. Lane 1, normal cells; lane 2, cells UV-irradiated at 5 J/m^2; lane 3, cells UV-irradiated at 10 J/m^2.

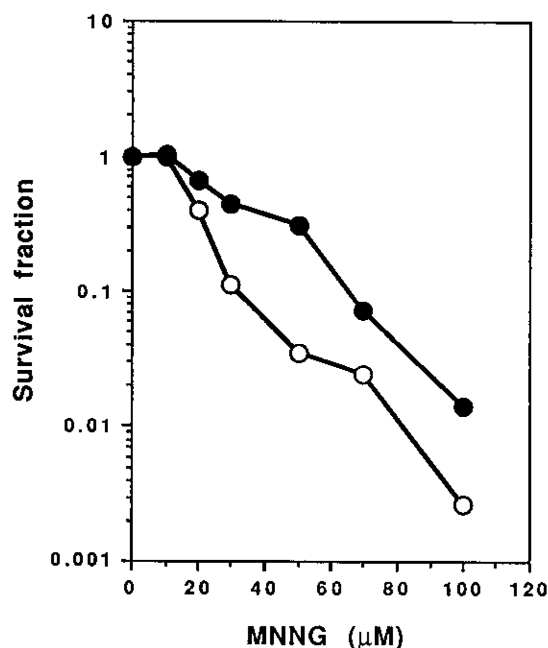

Fig. 5. Sensitivity to MNNG of H4IIE cells with or without UV irradiation. H4IIE cells UV-irradiated at 5 J/m^2 and those without irradiation were incubated for 48 h prior to exposure to various concentrations of MNNG. After incubation for 10 to 14 days, the number of colonies was counted. (○) normal H4IIE cells (control); (●) H4IIE cells UV-irradiated at 5 J/m^2.

than the control. There was no increase in amounts of a β-tubulin transcript measured using the same filter. Thus, it is likely that the increase in methyltransferase activity in the treated cells is due to an increased synthesis of messenger RNA for the enzyme.

MNNG sensitivity of UV-irradiated cells To determine the biological consequence of induction of the enzyme, the sensitivity to MNNG was determined with cells pre-exposed to UV. H4IIE cells were irradiated at 5 J/m^2, incubated for 48 h and then treated with various concentrations of MNNG. After removal of the agent, the cells were incubated in fresh medium for 10 to 14 days. Non-irradiated cells were treated in a similar manner. Fig. 5 shows that the UV-irradiated cells are significantly more resistant to MNNG than are non-irradiated cells. Thus, there is a correlation between the level of the enzyme activity and the enhancement of cell survival.

DISCUSSION

Alkylation of DNA at the O^6 position of guanine is regarded as one of the most critical events leading to

induction of mutations and cancers in organisms.[1, 26) Once O^6-methylguanine is formed, it can pair with thymine during DNA replication, resulting in conversion of the guanine-cytosine pair to the adenine-thymine pair in DNA.[3] Such mutations are often found in the DNA sequences of organisms exposed to relatively low doses of alkylating agents,[27] and it has been demonstrated that mammary tumors of rats, induced by injection of MNU, carry this type of mutation in the Ha-*ras*-1 gene.[4]

To counteract such effects, organisms possess a mechanism to repair O^6-methylguanine in DNA.[28] An enzyme, O^6-methylguanine-DNA methyltransferase, is present in organisms from bacteria to human cells, and appears to be responsible for preventing the occurrence of such mutations. The enzyme transfers methyl groups from O^6-methylguanine and other methylated moieties of the DNA to its own molecule, thereby repairing DNA lesions in a single-step reaction.[29]

O^6-Methylguanine-DNA methyltransferase is unique in many respects, among which the most notable is that the enzyme acts only once to fulfill its function. Once a methyl group is transferred to the enzyme molecule, it remains there and the methylated form of enzyme loses its catalytic activity. Thus, unlike ordinary enzymes, the action of methyltransferase is stoichiometric, and organisms require relatively large amounts of methyltransferase enzyme to repair DNA lesions. Indeed, many bacteria possess efficient systems to adjust the amounts of methyltransferase in response to levels of external DNA-damaging agents. In *E. coli* and *B. subtilis* cells, there are two known molecular species of methyltransferase, one constitutive and the other inducible. It is likely that the constitutive enzyme may handle a relatively small amount of DNA lesions formed under normal conditions, while the induced system would function under conditions where large amounts of lesions are produced.

The constitutive levels of methyltransferase in mammalian cells are relatively high as compared with those of bacteria. It was estimated that a HeLa cell contains about 5×10^4 enzyme molecules while the content in *E. coli* cell is less than 100 molecules per cell.[16] Nevertheless, the methyltransferase activity in rat hepatoma cells was found to increase considerably when the cells were exposed to UV and γ-rays.[17, 18]

In the present work we confirmed this using rat hepatoma cells. There was an increase in the enzyme activity with an enhanced expression of the gene, which is otherwise constitutively expressed in normal cells. Very recently, Laval[30] reported that about 5-fold increases of transcription of O^6-methylguanine-DNA methyltransferase gene occurred in rat hepatoma cells treated with γ-rays, *cis*-dichlorodiammine platinum II or 2-methyl-9-hydroxyellipticinium and this is in accord with our present finding. Potter *et al.*[31] reported that treatment with 2-acetylaminofluorene led to a 10-fold increase in the methyltransferase transcript in livers of adult rats. Thus, transcriptional activation occurs not only in cultured hepatoma cells but also in tissues of living animals.

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