Formation of O^6,7-Dimethylguanine Residues in Calf Thymus Deoxyribonucleic Acid Treated with Carcinogenic *N*-Methyl-*N*-nitrosourea *in Vitro*

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Treatment of calf thymus deoxyribonucleic acid (DNA) *in vitro* with a methylating carcinogen, *N*-methyl-*N*-nitrosourea (MNU), in phosphate buffer (pH 7.2) resulted in formation of O^6,7-dimethylguanine residues in DNA besides the well-known methylated DNA adducts, 7-methylguanine, O^6-methylguanine and 3-methyladenine. The product ratio (%) of O^6,7-dimethylguanine versus 7-methylguanine was 0.32 after one MNU treatment. The significance of formation of O^6,7-dimethylguanine residues in DNA is discussed briefly in relation to the carcinogenicity of MNU.

Keywords DNA damage; O^6,7-dimethylguanine; O^6-methylguanine; 7-methylguanine; DNA; methylation; *N*-methyl-*N*-nitrosourea; carcinogen; repair

Introduction

It is well known that the treatment of deoxyribonucleic acid (DNA) with methylating carcinogens *in vitro* and *in vivo* results in formation of varieties of methylated nucleobase and phosphate residues in DNA.[1] Among these methylated products, O^6-methylguanine and O^4-methylthymine residues are considered to cause the DNA damage most responsible for induction of mutation and/or cancer.[2,3] In our previous study on the reactivity of N-9 substituted O^6-methylguanine derivatives we revealed that their N-7 position is susceptible to further methylation resulting in formation of O^6,7-dimethylguanine derivatives.[4] In this paper we report the formation of O^6,7-dimethylguanine residues in calf thymus DNA treated with a carcinogenic *N*-methyl-*N*-nitrosourea (MNU) *in vitro*.

Experimental

Preparation of O^6,7-Dimethylguanine Our previously reported procedure[4] was modified. Briefly, O^6-methylguanine[5] was treated with CH_3I–K_2CO_3 in dimethylformamide to form a mixture of O^6,7-dimethylguanine and O^6,9-dimethylguanine. O^6,7-Dimethylguanine thus formed was separated by aluminum column chromatography eluted with $CHCl_3$: MeOH = 5 : 2. mp 252—253 °C. ^{1}H-NMR (DMSO-d_6) δ: 3.83 (s, 3H, N-CH_3), 3.97 (s, 3H, O-CH_3), 6.06 (br s, 2H, NH_2), 7.99 (s, 1H, H-8). UV λ_{max} nm (ε): 235 (sh) (6300) and 287 (11300) (pH 1), 240 (sh) (7500) and 288 (6700) (H_2O and pH 12). *Anal.* Calcd for $C_7H_9N_5O$: C, 46.93; H, 5.03; N, 39.11. Found: C, 46.86; H, 5.06; N, 39.13. MS *m/z*: 179 (M^+).

Other Materials 7-Methylguanine, 3-methyladenine and calf thymus DNA were purchased from Sigma Co., Ltd. O^6-Methylguanine was prepared by the method reported.[5]

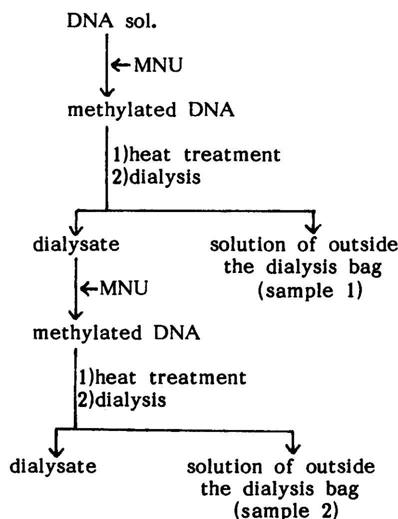

Fig. 1. Protocol for the Experiment

Treatment of DNA with MNU, and Separation of Methylated Purines Fifty-five mg of MNU dissolved in 200 μl of dimethyl sulfoxide (DMSO) was added to a DNA solution (10 mg of calf thymus DNA in 10 ml of 80 mM phosphate buffer (pH 7.2)). The mixture was incubated at 37 °C for 3 h. Then, 20 ml of cold EtOH was added to the mixture and the resulting fibrous DNA was collected by centrifugation (3000 rpm, 10 min). After the DNA was re-dissolved in 10 ml of a solution (10 mM phosphate buffer (pH 7.2)–100 mM NaCl), it was heated at 100 °C for 30 min. Then the DNA solution was dialyzed against 300 ml of H_2O at 4 °C for 6 h, and the solution of outside the dialysis bag was collected and lyophilized. The lyophilizate was dissolved in a small amount of H_2O and was used as a sample (sample 1) for high-performance liquid chromatography (HPLC) analysis. The dialysate (DNA solution in the dialysis bag) was lyophilized and dissolved in 10 ml of 80 mM phosphate buffer (pH 7.2). To this solution, 55 mg of MNU in 200 μl of DMSO was again added and the mixture was incubated at 37 °C for 3 h. After repeating the above procedure, a sample (sample 2) was obtained as the lyophilizate of the solution of outside the dialysis bag. The experimental protocol is shown in Fig. 1.

Analyses of Methylated Purines A Shimadzu LC-9A HPLC system equipped with photodiode ultraviolet (UV) detector was employed. The column was TSK-gel ODS 80TM (4.6 × 250 mm) and the solvent system was 10 mM NaH_2PO_4–15% CH_3OH at a flow rate of 0.7 ml/min. The yields of the products were calculated from their UV intensity and ε value.

Results and Discussion

Figure 2 shows the HPLC chromatogram of sample 1. Large peaks were observed around retention time (t_R) of 8 to 14 min and two small peaks with longer t_R followed. Peaks 1 and 2 were those of 3-methyladenine and 7-methylguanine, respectively. Peak 3 observed at t_R 51 min was identified as O^6,7-dimethylguanine by comparison of the t_R and UV absorption with those of an authentic sample. UV spectra of peak 3 and its authentic sample are shown in Fig. 3. The other small peak (peak 4) observed

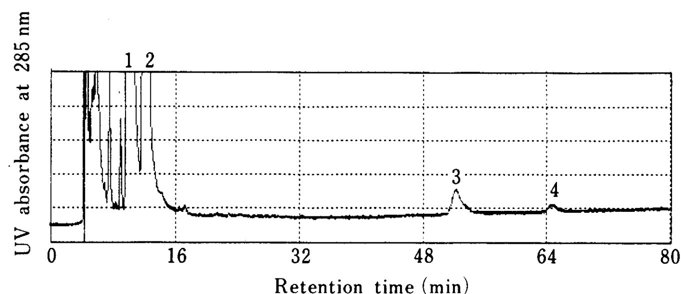

Fig. 2. Analysis of Methylated Purines
HPLC chromatogram of sample 1.

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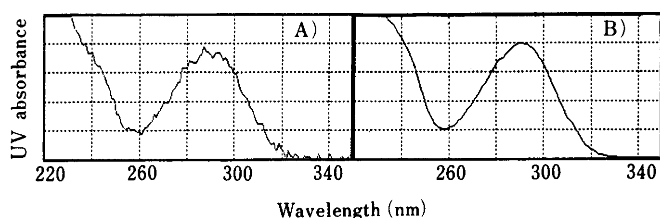

Fig. 3. UV Spectrum of O^6,7-Dimethylguanine

(A) Peak 3 in Fig. 2., (B) authentic sample.

TABLE I. Product Ratio of Methylated Purines[a]

Product	Ratio (%)	
	Sample 1	Sample 2
7-Methylguanine	100	100
O^6,7-Dimethylguanine	0.34	0.92
3-Methyladenine	12	11
O^6-Methylguanine	(9.4)[b]	

a) Expressed as % of 7-methylguanine. *b*) Determined separately by acid hydrolysis of the dialysate.

after peak 3 was not identified. The product ratio of methylated purines is shown in Table I. The percent ratio of O^6,7-dimethylguanine versus 7-methylguanine was 0.34 after the first MNU treatment (sample 1), and the ratio was increased to 0.92 by the second MNU treatment (sample 2). Since O^6,7-dimethylguanine is formed by the N-7 methylation of O^6-methylguanine residues,[4] the increased rate of O^6,7-dimethylguanine is due to the accumulated O^6-methylguanine residues in DNA by the first MNU treatment.

The existence of several unidentified minor components in DNA treated with methylating carcinogens was reported.[6] In this work we formulated the structure of one of them. The identification of O^6,7-dimethylguanine residues in cellular DNA treated with MNU is in progress.

The biological significance of O^6,7-dimethylguanine is an interesting subject to study, because the substance includes two well-known modifications, methylations at the O-6 and N-7 positions. O^6-Methylguanine residues in DNA are considered to cause the DNA damage most responsible for mutation and/or cancer, and are known to be reparable by an enzyme, O^6-methylguanine-DNA methyltransferase.[7] 7-Methylguanine residues are considered to cause damage related to the cell killing, and are known to be reparable by the enzyme, DNA glycosylase.[7] Could O^6,7-dimethylguanine formed in cellular DNA be repaired by these repair enzymes? If not, this modified portion would be a serious detriment in terms of initiation of mutation and/or cancer, even if its amount were extremely low.

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