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DEFICIENCY OF 8-HYDROXYGUANINE DNA ENDONUCLEASE ACTIVITY AND ACCUMULATION OF THE 8-HYDROXYGUANINE IN MUTATOR MUTANT (*MUTM*) OF *ESCHERICHIA COLI*

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Summary: In this report, we have characterized *mutM* mutant with respect to its ability to repair 8-hydroxyguanine (oh^{8}Gua) in DNA. The oh^{8}Gua DNA endonuclease activity in *mutM* strain was minimal as compared with that in the wild-type cells. The presence of oh^{8}Gua in DNA of *mutM* was 6-fold that of the wild-type strain corresponding to a characteristically higher frequency of G•C → T•A transversions in this mutant strain. These results suggest that mutator phenotype of *mutM* is at least partially due to a spontaneous accumulation of oh^{8}Gua resulting from a greatly reduced oh^{8}Gua DNA endonuclease activity.

Oxygen radicals are produced by cellular metabolism as well as ionizing radiation and environmental carcinogens (1). Oxygen radicals react with guanosine in DNA to generate 8-hydroxyguanine (oh^{8}Gua, 7,8-dihydro-8-oxoguanine, see review in ref. 2). In both *in vitro* and *in vivo* experiments, it has been shown that during DNA synthesis, the oh^{8}Gua causes misreading of DNA template resulting in G•C → T•A transversions in *E. coli* (3-6), and mainly causes G•C → T•A transversions in NIH3T3 cells (7).

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In *E. coli*, several mutator mutants have been isolated and the genes and their products, which are involved in the repair of oh^{8}Gua, have been characterized (8, 9). We previously isolated one of the DNA repair enzymes for the oh^{8}Gua, namely oh^{8}Gua DNA endonuclease which with respect to substrate specificity was shown to be the same as the formamidopyrimidine DNA glycosylase (FPG protein) coded by *mutM* gene. (8, 10). *MutM* mutant was originally characterized as a mutator phenotype with a high frequency of G•C → T•A transversions (11). However, adequate information has not been available to examine the relationship between the high mutability and oh^{8}Gua metabolism in *mutM* strain. In the present study, we report that an accumulation of oh^{8}Gua in *mutM* strain is associated with a greatly reduced oh^{8}Gua endonuclease activity and is very likely to be responsible, at least in part, for the high spontaneous frequency of G•C → T•A transversions as compaired to the normal wild-type *E. coli* strain.

Materials and Methods

Oligonucleotides

The sequences of oligodeoxynucleotides (21-mers) used as a substrate for the assay are 5'-CAGCCAATCAG^{OH}TGCACCATCC-3' and 5'-GGATGGTGCACTGATTGGCTG-3' (G^{OH} represents oh^{8}Gua). Preparation of oligonucleotides as a substrate for the assay has been described (12). Briefly, the oligonucleotide containing a single oh^{8}Gua at the specific site was synthesized by the method described previously (13) and another oligonucleotide (without oh^{8}Gua) was synthesized by phosphoramidite method using DNA synthesizer Model 380A (Applied Biosystems). oh^{8}Gua-containing oligonucleotide was labeled with [γ-^{32}P]ATP (Amersham) at the 5' termini using MEGALABEL (TaKaRa). This 5'-end labeled oligonucleotide was annealed with its complementary oligonucleotide at 65°C for 10 min in 10 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.5) and cooled at room temperature overnight. This double stranded oligonucleotide was used as a substrate for endonuclease nicking assay.

Bacterial strains

E. coli strains, CC104 (*ara, Δ(gpt-lac)$_5$, rpsL* [*F'lacI378, lacZ461, proA$^+$B$^+$*]) and CC105 strain which is identical to CC104 except for the

mutM mutation were a kind gift from Dr. J. H. Miller of University of California, Los Angeles.

Preparation of cell lysate

Bacterial cells were grown aerobically at 37°C overnight in LB medium (1 % tryptone, 0.5 % yeast extracts and 1 % NaCl). After the cells were harvested by centrifugation, cells were washed twice with 5 ml of PBS (Phsphate Buffered Saline) and resuspended with 1 ml of PBS followed by sonication for 1 min using SONORE 150S (UMEDA Electronic Co. Ltd.). After centrifugation to remove the cell debris, the supernatants were used as the cell lysate.

Endonuclease nicking assay

Endonuclease nicking assay was carried out as described by Chung *et al.* (12). Cell lysates (2 μg protein contents) were incubated with 100 fmole of 5'-end labeled double stranded oligonucleotide in 20 μl of 50 mM Tris-HCl pH 7.5, 50 mM KCl and 2 mM EDTA at room temperature for 30 min. Then the labeled-oligonucleotides in reaction mixture were purified by phenol/chloroform extraction and precipitated by adding 3 volumes of ethanol and then applied to 20 % PAGE (Polyacrilamide Gel Electrophoresis) containing 7 M Urea to analyze the cleaved DNA fragments.

Deoxyribonucleosides analysis

The method for analysis of deoxyribonucleosides was described previously (14). DNA was extracted from 5 ml of bacterial overnight cultures using DNA extraction kit 'GENOMIX' from TALENT (Italy) without phenol extraction. The DNA in 20 mM sodium acetate buffer (pH 4.8) was digested by 4 μg of nuclease P1 (Yamasa Shoyu, Chiba) at 37°C for 1 hr and then treated with 1.3 units of Bacterial Alkaline Phosphatase Type III (SIGMA Chemicals, St. Louis, MO) in 0.1 M Tris-HCl (pH. 7.5) at 37°C for 1 hr. The resulting deoxynucleosides mixtures were analyzed by HPLC-ECD system (15). The molar ratio of 8-hydroxydeoxyguanosine (oh^8dG) to deoxyguanosine (dG) was determined based on the profile of the authentic oh^8dG with the EC detector and UV-absorbance at A_{260} of dG. On the incubation of the DNA sample with *E. coli* alkaline phosphatase under the conditions described above, the content of oh^8Gua increased at the ratio of 3 ± 1 residues/10^6 dG/hr (14). Therefore, this blank value of oh^8Gua was subtracted from the observed data based on the actual incubation time. In Table I, the observed data were 3.7 ± 1.4 for CC104 (wild type) and 8.5 ± 0.93 for CC105 (*mutM* strain).

Results and Discussion

The results described here are the first evidence for the characterization of *mutM* strain in relation to the repair of oh^8Gua.

Table I. Accumulation of oh^{8}Gua in DNA in *mutM* and wild type strains

Strain	oh^{8}Gua in DNA oh^{8}dG per 10^6 dG
CC104	0.93[a] ± 0.87
CC105 (*mutM*)	5.5[a] ± 0.75

[a] Values represent the mean ± standard deviation from three independent cultures.

As mentioned above, *mutM* was originally characterized as a mutator strain that specifically leads to G•C → T•A transversions with higher mutation frequency than that of normal strain (11). One explanation for such high spontaneous mutation frequency in *mutM* might be the accumulation of oh^{8}Gua in DNA due to the deficiency of oh^{8}Gua endonuclease activity. In order to see the difference of oh^{8}Gua contents between *mutM* and wild-type cells, we have measured oh^{8}Gua in DNA in both type of cells by using the HPLC-EC detection system. As shown in Table I, 6-fold more oh^{8}Gua was detected in *mutM* cellular DNA than in the DNA from wild type cells.

In order to confirm that MutM protein actually works as the repair protein for the oh^{8}Gua DNA endonuclease, we have compared oh^{8}Gua DNA endonuclease activity in crude extracts of *mutM* cells and wild-type cells, using a synthetic oligonucleotide containing an oh^{8}Gua at a specific site as the substrate. As shown in Fig. 1, two distinct cleavage products which were driven by incision of the site of oh^{8}Gua residue were observed, when oligonucleotides containing an oh^{8}Gua were incubated with the cell extracts of wild-type cells. One product, 10-mer (3'-P) has been detected when incubated with the purified *E. coli* oh^{8}Gua DNA endonuclease (data not shown; 12). It has been shown previously that another product, 10-mer (3'-OH) is the dephospholyrated product of 10-mer (3'-P) (12). Such cleavage products

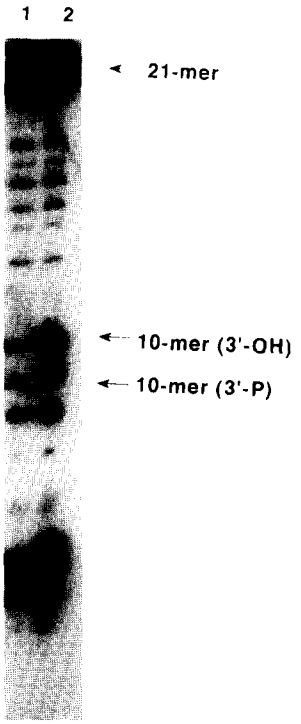

Fig. 1. The detection of oh^{8}Gua-endonuclease activity in *E. coli* strains. The products by treating the substrate DNA with the lysate of CC105 (*mutM*) are shown in lane 1 and with the lysate of CC104 (wild type) are shown in lane 2.

were not detected from the unmodified oligomer that contained normal Gua residue (data not shown). In contrast to the wild-type cell extract, such an endonuclease activity was barely detected in *mutM* cell extracts (Fig. 1). It should be noted that there is still a small amount of activity detectable in *mutM* extracts probably due to the endonuclease activity from endonuclease III in *E. coli* cells (12). This suggested that MutM protein could principally work as the oh^{8}Gua DNA endonuclease in *E. coli* cells. Although oh^{8}Gua may not be the only DNA lesion to explain the high mutability of *mutM* strain, it is unlikely that other types of DNA damage affect the mutability in *mutM* strain. In fact, the Fapy-DNA glycosylase defective strain which is the same as *mutM* mutant does not show unusual sensitivity to the treatment of alkylating agents which produce alkylguanine in DNA (16).

Recently it has been shown that natural substrate for MutY protein, which has been characterized as an glycosylase that removes the A from G•A mismatch (17), is not G•A mismatch but oh^{8}Gua•A mismatch (18). Interestingly, *mutM/mutY* double mutation leads to a far more increase of G•C → T•A transversion frequency than *mutM* and *mutY* single mutant (18). In addition, MutT protein encoded by the *mutT* gene specifically hydrolyzed oh^{8}dGTP in cellular nucleotide pool to keep the high fidelity of DNA synthesis (9).

We would like to conclude that the deficiency in oh^{8}Gua DNA endonuclease activity by *mutM* mutation leads to an accumulation of oh^{8}Gua residues in DNA and that these lesions are one of the main contributors to an increase in G•C → T•A transversion frequency. These observations are consistent with the phenotype of *mutM* strain predicted by the mutational specificity.

The pathways that lead to spontaneous mutations are not completely understood yet, and it is still uncertain which types of DNA damage cause spontaneous mutations. However, the evidence strongly suggests that the oh^{8}Gua generated physiologically is one of the major types of DNA damages responsible for spontaneous mutations. Finally we would like to mention that a similar work is carried out using mass spectral analysis by Tchou *et al.* of Department of Pharmacological Sciences, State University of New York at Stony Brook, New York.

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