Analysis of N-Methyl-N-nitrosourea-induced Mutations in a Shuttle Vector Plasmid Propagated in Mouse O6-Methylguanine-DNA Methyltransferase-deficient Cells in Comparison with Proficient Cells

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ABSTRACT

A shuttle vector plasmid, pYZ289, was constructed from the pZ189 plasmid and polyoma virus DNA. The plasmid contains a supF gene as a marker of mutation and can replicate in both Escherichia coli and mouse cells. The pYZ289 plasmids treated with N-methyl-N-nitrosourea were passed through mouse cells originating from skin tumors, which are either proficient (HL18) or deficient (HL8) in O6-methylguanine-DNA methyltransferase activity, and mutations in the supF gene were analyzed. In the repair-deficient HL8 cells, N-methyl-N-nitrosourea-treated pYZ289 showed lower plasmid survival and higher mutation frequency than in the repair-proficient HL18 cells. DNA sequence analysis in the mutated supF gene revealed that most mutations occurred in G:C base pairs (86% for HL8, 76% for HL18), and the frequency of G:C — A:T transition was higher in HL8 cells (69%) than in HL18 cells (35%). G:C — A:T transversions occurred more frequently in HL18 cells (31%) than in HL8 cells (12%). Mutations occurred frequently at the base pair positions of 2, 15 and 150 of supF gene in HL18 cells and at 169 in HL8 cells. Analysis of the bases neighboring the mutations appeared to be related to the mutability of the base pairs with the sequence of 5'-purine-G-G-3' being the most frequently mutated. These results show that the new pYZ289 plasmid is useful for the analysis of mutations and that a deficiency in O6-methylguanine-DNA methyltransferase enhances the N-methyl-N-nitrosourea-induced mutation with significant specificity.

INTRODUCTION

Alkylating agents such as MNU or MNNG can produce many kinds of adducts with bases in DNA (1). Among them, O6MeG has been thought to be the major lesion for mutagenesis and carcinogenesis because of its preferential pairing with thymine instead of cytosine, during the semiconservative DNA replication, leading to the G:C — A:T transition mutation (2–5). O6MeG in DNA is repaired by OMT (1, 2). This enzyme accepts the methyl group from DNA to its cysteine residue and becomes inactivated in a suicide reaction (1, 6).

Approximately 20% (19 of 93) in Ref. 7 and 5 of 40 in Ref. 8) of human tumor cell lines, 64% (7 of 11) of SV40-transformed human fibroblast cell lines (9) and 27% (7 of 26) of Epstein-Barr virus-transformed human lymphoblastoid cell lines (10) are defective or have extremely low OMT activity. These cells are hypersensitive to the alkylating agents and were termed Mer-/Mex- (9–11). Recently we reported that about 13% (6 of 47) of the murine tumor cell lines derived from UVB-induced tumors have the phenotype Mer-/Mex- (11). These results suggest that the decrease or deficiency in OMT activity is responsible for carcinogenesis. However, the precise mechanism of the role of OMT in carcinogenesis has not been elucidated.

In the MNU-induced rat mammary carcinomas, the H-ras or K-ras genes are activated by a G:C — A:T transition at the middle base of codon 12, encoding glutamic acid or aspartic acid, respectively, in place of glycine (12–14). Brown et al. (15) reported that 5 of 12 MNU-induced skin tumors and 13 of 28 MNNG-induced skin tumors in mice also have a G:C — A:T transition at the same position as that in H-ras gene. These data agree with the concept that the major lesion produced by MNU or MNNG treatment leading to mutagenesis and carcinogenesis is O6MeG, which may result in a G:C — A:T transition mutation by mispairing with thymine during DNA replication.

To examine what type of mutation occurs preferentially by the MNU treatment, and what role OMT plays in the MNU-induced mutagenesis in mouse cells, we constructed a novel shuttle vector plasmid, pYZ289, from a shuttle vector plasmid, pZ189, and polyoma virus DNA. Plasmid pZ189, that has been recently developed for assessing mutagenesis in mammalian cells (16–19), can replicate in both human and prokaryotic cells but not in mouse cells. Plasmid pYZ289 replicates in both mouse and Escherichia coli cells and carries a bacterial tyrosine suppressor tRNA gene (supF) as a marker of mutation.

We transfected pYZ289 plasmids treated with MNU in vitro to the mouse Mer+/Mex+ and Mer-/Mex- cells by electroporation, and induced them to replicate and be repaired in the cells. The plasmids were extracted and mutations in the supF gene were detected in E. coli cells, followed by DNA sequencing analysis. Frequency and type of the base substitution mutation in the MNU-treated plasmids were compared between Mer+/ Mex+ and Mer-/Mex- cells.

MATERIALS AND METHODS

Cells. Two mouse tumor cell lines (HL18, HL8) derived from UVB-light-induced tumors of specific pathogen-free hairless mice of the inbred strain HOS(HR/De) were used in this study. HL18 cells have high OMT activity (238 fmol/mg protein) and are resistant to MNU (D37 = 1.8 μm) and ACNU (D37 = 42 μm), while HL8 cells are OMT-deficient (2 fmol/mg protein) and hypersensitive to the agents (MNU, D37 = 0.2 μm; ACNU, D37 = 1.6 μm) (11).

Cells were cultured in Dulbecco’s modified minimum essential medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) at 37°C in a 5% CO2 atmosphere.

Plasmid Construction. A novel plasmid, pYZ289 (7.6 kilobases) (Fig. 1) replicates in both mouse and E. coli cells. This was constructed from pZ189 (16) and pPyBamHI which was developed by K. Segawa and was obtained from Japanese Cancer Research Resources Bank. Plasmid pPyBamHI contains the entire polyoma virus (A2 strain) genome in a PUC13 vector. A DNA fragment (2.4 kilobases) containing pBR327 replication origin, tyrosine suppressor tRNA gene (supF) and β-lactamase gene was obtained from pZ189 by BamHI
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RESULTS

Plasmid Survival and Mutagenesis. We obtained about $2.2 \times 10^4$ and $6 \times 10^2$ transformants on average from HL18 and HL8 cells, respectively, in one transfection experiment with untreated pYZ289. MNU-treated pYZ289 plasmids extracted from both HL18 and HL8 cells showed a dose-dependent decrease in the relative number of ampicillin-resistant colonies compared to the untreated control. Survival of the plasmids recovered from HL8 cells was much lower than that from HL18 cells (Fig. 2). This is consistent with the results of cell survival measured by colony-forming ability after treatment with MNU (data not shown) and ACNU (11) and host cell reactivation of herpes simplex virus treated with the alkylating agents (7, 9, 24).

MNU treatment of the plasmids enhanced the frequency of the mutant colonies (white or light blue) in a dose-dependent manner in both HL18 and HL8 cells. The frequency of spontaneous mutation was almost the same in HL18 and HL8 cells ($1.9 \times 10^{-3}$ and $2.4 \times 10^{-3}$, respectively), while the increase in mutation frequency in HL8 cells was much higher than in HL18 cells. At a concentration of 10 mM, more than 70% of the plasmids recovered from HL8 cells were mutant in each transfection experiment. At a concentration of 10 mM in HL18 cells and 2 mM in HL8 cells, the frequencies of MNU-induced mutations were about 10 times higher than those of spontaneous mutation ($23.8 \times 10^{-3}$ and $22.5 \times 10^{-3}$, respectively) (Fig. 3). We examined the nucleotide sequence changes in the supF gene treated with these concentrations of MNU.

Base Sequence Analysis. Fifty-nine and 54 independent supF mutant plasmids extracted from HL18 and HL8 cells, respectively, were analyzed by agarose gel electrophoresis and nucleotide sequencing. Deletions were found in 25% (15 of 59) and 22% (12 of 54) of the mutant plasmids from HL18 and HL8 cells, respectively. Other mutant plasmids contained base substitution mutations, of which about 60% were single, 10% were tandem, and 5% were tandem in the supF gene from HL18 and HL8 cells combined. There was no mutant containing 3 or more base substitutions in the supF gene. Most of them (76% from HL18 and 87% from HL8) occurred at G:C pairs. These are significantly ($P = 1.1 \times 10^{-5}$) higher than the frequency estimated by the random distribution in the supF nucleotide

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**Fig. 1.** Schematic diagram of the plasmid pYZ289 constructed from pZ189 and pPyBamHI. The BamHI-digested polyoma virus DNA sequence from pPyBamHI is represented by a *bold line*, containing the regions of the replication origin (ori), T-antigen, and viral protein (VP). Selected restriction sites are indicated: B, BamHI; E, EcoRI; kb, kilobase.

**Fig. 2.** Survival of MNU-treated pYZ289 plasmids propagated in HL18 (C) and HL8 (○) cells. The relative number of ampicillin-resistant bacterial colonies with MNU-treated plasmid compared with those of the untreated control after replication in both cells followed by transformation of E. coli, MBM7070, is shown. Points, mean value from 4–18 independent transfection experiments. The standard errors are smaller than the symbols for most points.

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Fig. 3. Mutation induced in MNU-treated pYZ289 plasmids propagated in HL18 (O) and HL8 (•) cells. The frequency of white and light blue colonies is shown based on 4–18 independent transfection experiments.

The relative sensitivity of HL18 and HL8 cells to MNU, indicating that the DNA repair mechanism efficiently functioned in the input plasmid DNA as well as in the chromosomal DNA. Repair of MNU-produced O6MeG in the plasmid by a cellular enzyme would play an important role in plasmid survival and the prevention of mutation.

The most frequent among base substitutions were G:C → A:T transitions in both HL18 and HL8 cells. The proportion of G:C → A:T transitions was much higher in the O6MT-deficient HL8 cells (69%) than in the HL18 cells (35%; \( P = 0.0005 \)). Repair of O6MeG mainly contributed to the decrease in the MNU-induced G:C → A:T transitions which were presumably due to thymine misincorporations at the opposite sites of O6MeG in the damaged DNA.

The majority of the base substitutions in the supF gene in HL18 cells, was transversions (63%), nearly one-half of them sequences (56%). The transition mutations were predominated in HL8 cells and were far more frequent than those in HL18 cells (\( P = 0.0001 \)) (Table 1). In particular, the frequency of G:C → A:T transitions was higher in HL8 cells than in HL18 cells (\( P = 0.0005 \)). In contrast, G:C → T:A transversions occurred more frequently in HL18 cells than in HL8 cells (\( P = 0.01 \)).

Mutational Spectra. Distribution of the base substitutions in the supF gene between base pairs 34 and 181 were analyzed (Fig. 4). The substituted bases were not randomly distributed and the positions were considerably different between HL18 and HL8 cells. The base substitutions at base pair 159 in HL18 cells and at base pair 169 in HL8 cells were significantly more frequent than in the other cell line (\( P = 0.013 \) and \( P = 0.02 \), respectively). Analysis of the neighboring bases of the substituted bases revealed that the middle base in the sequence of 5’-purines-G-G-3’ appeared to be more frequently mutated in HL8 than in HL18 cells. In the repair-proficient HL18 cells, there was no apparent specificity of the neighboring bases.

DISCUSSION

The survival of MNU-treated pYZ289 plasmids was lower and the MNU-induced mutation frequency was higher when they were transfected into the O6MT-deficient HL8 cells than in the O6MT-proficient HL18 cells. This is consistent with the relative sensitivity of HL18 and HL8 cells to MNU, indicating that the DNA repair mechanism efficiently functioned in the input plasmid DNA as well as in the chromosomal DNA. Repair of MNU-produced O6MeG in the plasmid by a cellular enzyme would play an important role in plasmid survival and the prevention of mutation.
being G:C → T:A substitutions. Previous reports have shown that the frequency of transversions in shuttle vector genes with MNU or ethynitrosourea treatment was only 10–35% (G:C → T:A transversions were less than 10%) in O'MT-proficient human cells (25–27). A similar low frequency of transversions was seen with the O'MT-deficient HL8 cells (25%). One possibility is that the mouse O'MT-proficient HL18 cells have extremely high O'MT activity, with a very efficient repair capacity of O'MeG. The HL8 strain was chosen since it had the highest O'MT activity among 47 strains established from mouse tumors (11), and appeared to be more resistant to alkylating agents than other strains. The low frequency of transitions with HL18 cells could be due to the efficient repair of O'MeG resulting in less G:C → A:T transitions. Another possible cause of the high frequency of G:C → A:T transversions may involve so-called “A” rule, preferential inserts of adenines opposite to the damaged bases (27–29).

Sikpi et al. (25) reported analyses of MNU-induced mutations in the shuttle vector pZ189 with human Mex* and Mex- cells. Transitions, G:C → A:T, were predominant in both Mex* and Mex- cells at almost the same frequencies and G:C → T:A transversions were very few. Sites of the base substitutions were considerably different from our data. One basic difference in the experimental system, we noticed, was that the Mex* and Mex- strains used by Sikpi et al. were not related, whereas we used isogenic strains. Dubridge et al. (26) analyzed MNU-induced mutations by using human embryonic kidney cells with an Epstein-Barr virus-lacI shuttle vector, and showed that more than 90% of the base substitutions were G:C → A:T transitions.

The G:C pairs at base pairs 123 and 159 in the supF gene were found to be at highly mutable positions in O'MT-proficient HL18 cells. Sikpi et al. (25) did not detect a mutation at base pair 159, but they found high frequencies of mutation at base pair 123 in both human Mex* and Mex- cells. This site is also an UV-induced mutation hot spot in human fibroblasts (22, 30). Guanine at position 160 is a hot spot of G:C → A:T transition in HL18 cells and cytosine at position 169 is a hot spot of G:C → A:T transition in O'MT-deficient HL8 cells. Both positions were significantly more mutable in one strain than the other. The difference in hot spot positions between O'MT-proficient and -deficient cells may be due to differences in the MNU concentration, which was adjusted to yield the same mutation frequencies. The real hot spot should be base pair 169 which was significantly higher in HL8 than in HL18.

In O'MT-proficient HL18 cells, the damaged base pair 169, O'MeG, must be repaired efficiently, yielding less base substitutions than in the O'MT-deficient HL8 cells. If the repair is preferential in the transcribing strands as reported in UV-induced mutagenesis (31, 32), the sequence opposite cytosine at base pair 169 may represent the transcribed region of the supF gene. The specificity with base pair 169 may have another cause involving the neighboring bases.

In O'MT-deficient HL8 cells, the mutation occurred most frequently in guanine in the middle of 5'-purine-G-G-3' sequences. Dubridge et al. (26) also reported that the MNU-induced hot spots were at guanine residues with guanines at the 5' side. G:C → A:T transitions at the center of the DNA sequence of the 12th codon (GGA) in c-H-ras genes were detected in MNU-induced rat mammary carcinomas and mouse skin tumors (12–15). This agrees with our results, although the 3' bases of the mutated guanines in the c-H-ras genes were not guanine, but adenine.

This study showed that pYZ289 constructed from pZ189 and polyoma virus DNA replicated in both mouse and E. coli cells and it was very useful for detailed analyses of MNU-induced mutation. We believe that pYZ289 should also be suitable for analyzing mutations caused by other environmental mutagens in rodent cells. During the preparation of this report, a similar shuttle vector plasmid was constructed independently by Zernik-Kobak et al. (33).

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