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Mini Review

ROLES OF DNA REPAIR METHYLTRANSFERASE IN MUTAGENESIS AND CARCINOGENESIS

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Summary Alkylation of DNA at the O^6-position of guanine is one of the most critical events leading to induction of mutation as well as cancer. An enzyme, O^6-methylguanine-DNA methyltransferase, is present in various 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. To elucidate the role of methyltransferase in preventing cancer, animal models with altered levels of enzyme activity were generated. Transgenic mice carrying extra copies of the foreign methyltransferase gene showed a decreased susceptibility to alkylating carcinogens, with regard to tumor formation. By means of gene targeting, mouse lines defective in both alleles of the methyltransferase gene were established. Administration of methylnitrosourea to these gene-targeted mice led to early death while normal mice treated in the same manner showed no untoward effects. Numerous tumors were formed in the gene-defective mice exposed to a low dose of methylnitrosourea, while none or only few tumors were induced in the methyltransferase-proficient mice. It seems apparent that the DNA repair methyltransferase plays an important role in lowering a risk of occurrence of cancer in organisms.

Key Words mutation, tumor, DNA damage, DNA repair, alkylating agent

Introduction

Exposure of cells to alkylating agents produces varius alkylated adducts in DNA. Among many of the alkylated bases formed, O^6-methylguanine appears to be a major pre-mutagenic lesion in organisms (Loveless, 1969; Strauss *et al.*, 1975). During DNA replication, O^6-methylguanine pairs with cytosine as well as thymine

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and, as a result, the guanine-cytosine pair can be converted to the adenine-thymine pair (Coulondre and Miller, 1977). Such mutations are frequent in DNA sequences of organisms exposed to relatively low levels of alkylating agents (Ito *et al.*, 1994), and it has been demonstrated that mammary tumors of rats, as induced by injection of methylnitrosourea (MNU), carry this type of mutation in the *Ha-ras*-1 gene (Sukumar *et al.*, 1983).

To counteract such effects, organisms possess a mechanism to repair O^6-methylguanine in DNA. An enzyme, O^6-methylguanine-DNA methyltransferase, is present in various organisms, and appears to be responsible for preventing the occurrence of such mutations (Demple *et al.*, 1982; Sekiguchi *et al.*, 1996). 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. Since this reaction irreversibly inactivates the enzyme, the capacity for O^6-methylguanine adduct repair depends on the number of active enzyme molecules per cell (Ishibashi *et al.*, 1994a, b).

Cellular contents of methyltransferase protein vary with tissues, and it was pointed out that more tumors are formed in tissues with less methyltransferase activity, in alkylnitrosourea-administered animals (Kleihues and Margison, 1974; Goth and Rajewsky, 1974; Gerson *et al.*, 1986). Some human tumor-derived cell lines are hypersensitive to alkylating agents, and these cell lines, termed Mer^- or Mex^-, have little or no methyltransferase activity (Day *et al.*, 1980; Sklar and Strauss, 1981; Yarosh *et al.*, 1983). It was suspected that this methyltransferase deficiency might possibly explain the frequent occurrence of tumors, in certain cases, even though methyltransferase deficiency is in most cases the consequence of cellular transformation.

To elucidate the roles of methyltransferase in preventing occurrence of cancer, appropriate animal models with altered levels of the enzyme activity are needed. We and several other investigators generated transgenic mice with elevated levels of methyltransferase activity and found that such animals are more resistant to tumor induction by alkylating agents, as compared with normal mice (Nakatsuru *et al.*, 1993; Dumenco *et al.*, 1993; Zaidi *et al.*, 1995). We also obtained methyltransferase-deficient mice by gene targeting and demonstrated that such mice are extremely hypersensitive to alkylating carcinogens (Tsuzuki *et al.*, 1996; Sakumi *et al.*, 1997; Iwakuma *et al.*, 1997). We summarize here an update on understanding of roles of methyltransferase in mutagenesis and carcinogenesis.

Structure and Function of Methyltransferase

Many organisms, from bacteria to humans, carry the enzyme, O^6-methylguanine-DNA methyltransferase. The structure and function of methyltransferase have been studied extensively in *Escherichia coli* (Nakabeppu *et al.*, 1985; Demple *et al.*, 1985; Margison *et al.*, 1985; LeMotte and Walker, 1985; Wilkinson *et al.*, 1989). We will first review briefly characteristics of the enzyme from this bacterium.

E. coli possesses two types of methyltransferase, a 39-kDa Ada protein and a 19-kDa Ogt protein, coded by *ada* and *ogt* genes, respectively. The Ada protein carries two distinct methyltransferase activities, one to transfer a methyl group from methylphosphotriester and the other to transfer a methyl group from either O^6-methylguanine or O^4-methylthymine of methylated DNA. These two activities reside on N-terminal and C-terminal halves of the Ada protein, and the Ogt protein carries only the region corresponding to the latter (Fig. 1). Although methylation of the phosphate backbone of DNA has no major biological effect, methyl transfer to the N-terminal half of the Ada protein causes activation of this protein, as a transcriptional regulator, thereby overproducing Ada protein and related enzymes in response to externally administered alkylating agents (for review, Sekiguchi and Nakabeppu, 1987; Lindahl *et al.*, 1988).

The amino acid sequence of the Ogt protein has a striking homology with the C-terminal region of the Ada protein. The Ogt protein repairs O^6-methylguanine and O^4-methylthymine and thus is structually and functionally similar to the C-terminal half of the Ada protein. Eukaryotic cells contain a single type of methyltransferase, which is similar to the *E. coli* Ogt protein. As amounts of Ogt-type proteins are not increased after treatment with alkylating agents, these proteins are regarded as constitutive enzymes.

Cloning of cDNA for human methyltransferase was achieved in 1990 by three groups of investigators, each using different strategies (Hayakawa *et al.*, 1990; Rydberg *et al.*, 1990; Tano *et al.*, 1990). The amino acid sequence of the purified protein was in complete agreement with that deduced from the nucleotide sequence of the cloned cDNA (Koike *et al.*, 1990). Using a probe derived from the human sequence, cDNAs for mouse, rat, rabbit and hamster enzymes have been isolated (Sakumi *et al.*, 1991; Potter *et al.*, 1991; Rahden-Staron and Laval, 1991; Shiraishi *et al.*, 1992; Shiota *et al.*, 1992; Rafferty *et al.*, 1992; Iyama *et al.*, 1994). The nucleotide sequences of the cDNAs revealed a high homology over the protein as a whole and sizes of the mammalian enzymes are slightly larger than size of the *E. coli* Ogt protein (Fig. 1).

Inspection of these sequences revealed that the cysteine residue accepting a methyl group from the methylated bases of DNA is located within the sequence of Pro-Cys-His-Arg (see Fig. 1). To elucidate the significance of a highly conserved amino acid sequence of the methyltransferase protein, amino acid substitutions were introduced by site-directed mutagenesis of cloned cDNA for human methyltransferase (Chueh *et al.*, 1992). More extensive analyses were made with the *E. coli* Ogt protein, and evidence was obtained to show that the sequence Pro-Cys-His-Arg is a *sine qua non* for methyltransferase to exert its function (Ihara *et al.*, 1994).

Functions of Methyltransferase in Preventing Mutation

E. coli mutant strains that lack either one or both of the *ada* and *ogt* genes

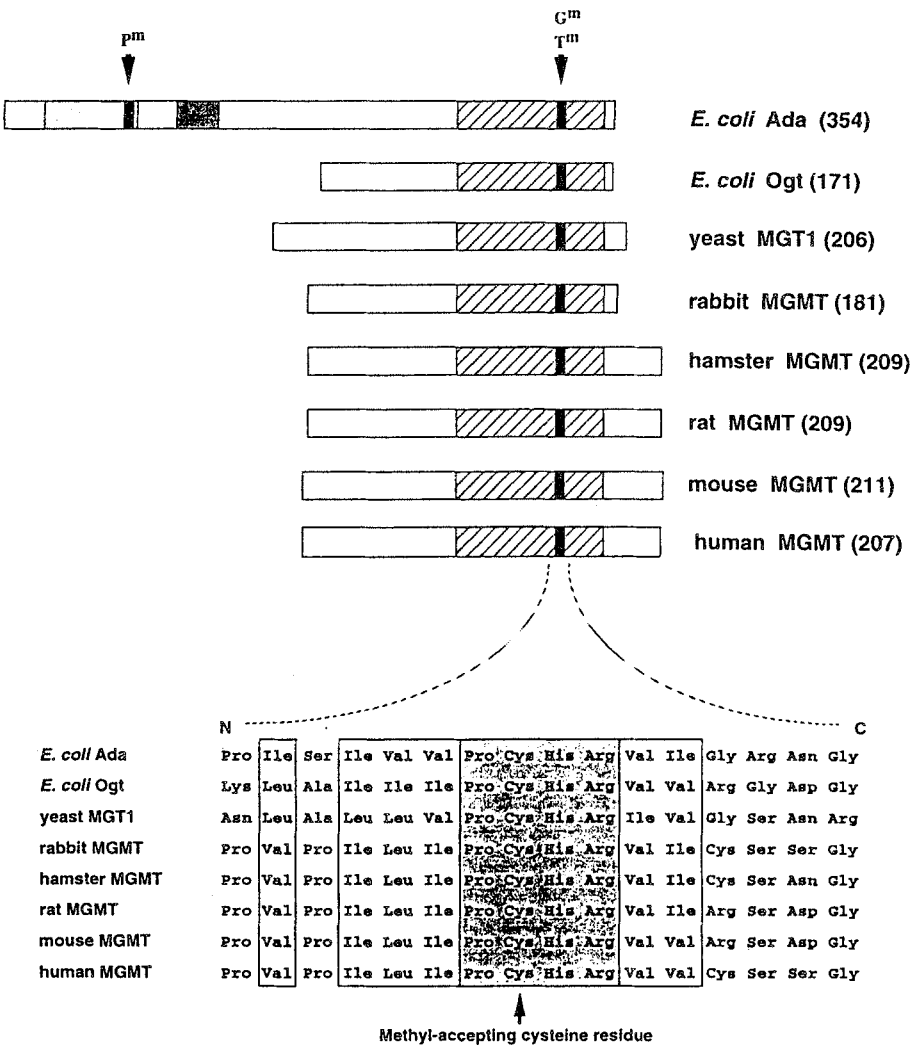

Fig. 1. Comparison of structures of DNA repair methyltransferases from various organisms. The upper panel shows the overall structures with methyl acceptor sites. Numbers in parenthesis indicate the numbers of amino acid residues of the proteins. The lower panel indicates amino acid sequences around the methyl acceptor sites.

were constructed (Takano *et al.*, 1991). In either the *ada*$^+$ or *ada*$^-$ background, the *ogt* mutation had no effect on cell survival after *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine (MNNG) treatment. On the other hand, *ada*$^-$ *ogt*$^-$ cells were more prone to mutation as compared to the *ada*$^-$ *ogt*$^+$ cells exposed to MNNG. Although the frequency of spontaneous mutation of cells defective in either one or

both of the genes was the same, introduction of the *ogt*$^+$ plasmid into the cells produced a 2 to 3-fold decrease in the frequency of spontaneous mutation. Rebeck and Samson (1991) found that more mutations arise in non-dividing *ada*$^-$ *ogt*$^-$ cells than in wild type cells. Thus, methyltransferase appears to function so as to eliminate pre-mutagenic DNA lesions not only from cells exposed to alkylating agents but also from cells proliferating in the absence of these agents.

Types of mutations occurring in cells exposed to MNNG have been analyzed, using an assay for forward mutations arising in a specific gene sequence (Ito *et al.*, 1994). Mutations recovered from wildtype strains were predominantly G•C to A•T transitions, located at several "hot" spots in the target gene. Although *ada*$^-$ *ogt*$^-$ cells showed hypersensitivity to the alkylating agent in terms of mutagenic and cell-killing effects, the type and site distribution of the mutations recovered from the mutants were similar to those observed with the wild type cells.

Some tumor-derived cell lines are hypersensitive to alkylating agents and are unable to reactivate virus pre-exposed to alkylating agents (Day *et al.*, 1980; Sklar and Strauss, 1981; Yarosh *et al.*, 1983). These Mer$^-$ (Mex$^-$) cells have little or no methyltransferase activity, but the molecular mechanisms underlying this Mer$^-$ character are not fully understood. No or only a little mRNA for methyltransferase was detected in Mer$^-$ cells, though there is no gross difference in coding and promoter regions of the methyltransferase gene in Mer$^+$ and Mer$^-$ cells (Pieper *et al.*, 1990; Tano *et al.*, 1990; Rydberg *et al.*, 1990; Nakatsu *et al.*, 1993). It can, therefore, be assumed that these Mer$^-$ cells are defective in transcriptional control, and this leads to the question of whether or not Mer$^-$ cells have altered expressions in genes other than that for methyltransferase itself.

For elucidation, cell lines defective particularly in the methyltransferase gene were developed, using gene targeting techniques. The cell lines have a sequence alteration in the defined region of the *MGMT* alleles and are totally devoid of methyltransferase protein, as confirmed in immunological and enzymatic assays (Tominaga *et al.*, 1997). *MGMT*$^{-/-}$ cells showed an increased mutation frequency as compared with the parental *MGMT*$^{+/+}$ cells when treated with a low level of MNNG (Fig. 2). This assay was made by scoring 6-thioguanine-resistant colonies which arose by forward mutations in the *HPRT* locus. The level of mutability of *MGMT*$^{-/-}$ cells was much the same as that obtained with Mer$^-$ cells, whose forward mutation frequency was measured using the same genetic marker (Domoradzki *et al.*, 1984).

Decreased colony-forming ability of cells after exposure to alkylating agents is another notable feature of Mer$^-$ cells (Day *et al.*, 1980). The LD_{37} of *MGMT*$^{+/+}$, *MGMT*$^{+/-}$ and *MGMT*$^{-/-}$ cells was 11.3, 7.3 and 0.11 μM MNNG, respectively, and similar degrees of sensitivity were obtained with MNU (Tominaga *et al.*, 1997). Thus, cells which are totally deficient in methyltransferase protein are 100 times more sensitive to alkylating agents than are methyltransferase-proficient cells, and this coincides with the sensitivity levels of Mer$^+$ and Mer$^-$ cells (Domor-

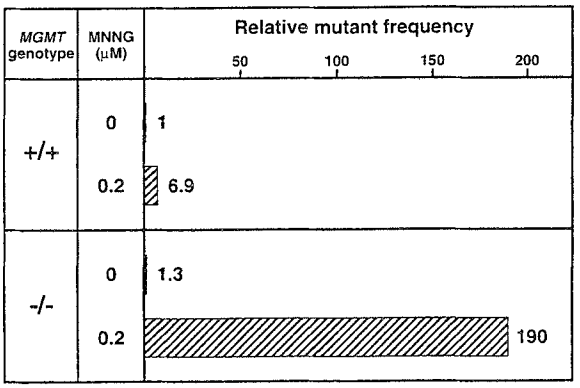

Fig. 2. Relative frequency of 6-thioguanine-resistant mutants in $MGMT^{+/+}$ and $MGMT^{-/-}$ cells after treatment with MNNG. Data were taken from Tominaga *et al.* (1997).

adzki *et al.*, 1984). We found that a single cell with a $MGMT^{+/-}$ background carries 1.7×10^4 methyltransferase molecules, while a $MGMT^{+/+}$ cell contains 2.4×10^4 methyltransferase molecules. This enzyme level in $MGMT^{+/-}$ cells (70% of $MGMT^{+/+}$ cell) clearly reflects the level of sensitivity of the heterozygous cell (65% of $MGMT^{+/+}$ cell), as expected from the finding that stoichiometric amounts of methyltransferase protein are needed for DNA repair (Olsson and Lindahl, 1980).

Lowered survival of methyltransferase-deficient cell lines after treatment with alkylating agents implies that O^6-methylguanine and, to a lesser extent, O^4-methylthymine are not only mutagenic but are also lethal. The toxicity of such lesions might be attributed to the inappropriate processing of mismatch repair. This was first suggested in studies with *E. coli dam*$^-$ strains, in which mutations in the mismatch recognition genes *mutS* and *mutL* confer protection against the toxicity of MNNG (Jones and Wagner, 1981; Karran and Marinus, 1982). Involvement of alkylated bases in mammalian cell lethality was deduced from the observation that nicks persisted in DNA of the Mer$^-$ cells that had been exposed to alkylating agents (Kalamegham *et al.*, 1988). Recent studies indicated that an acquired resistance (methylation tolerance) of Mer$^-$ cell lines is indeed associated with loss of capacity for mismatch repair (Branch *et al.*, 1993; Kat *et al.*, 1993). It has been proposed that the accumulation of alkylated bases in chromosomal DNA may provoke abortive mismatch repair, thereby leading to cell death. If a defect in the mismatch gene could be introduced into $MGMT^{-/-}$ cells, then one would have a pertinent tool for examining the cause of alkylation-induced cell death.

Tumorigenesis in Methyltransferase Gene-Disrupted Mice

To elucidate the roles of methyltransferase in carcinogenesis, appropriate animal models with altered levels of the enzyme activity are needed. This can be

achieved in two ways, one to generate transgenic mice carrying extra copies of the foreign methyltransferase gene with functional promoters and the other to produce methyltransferase-deficient mice by means of gene targeting. The latter is technically more difficult but would be more definitive regarding *in vivo* functions of the enzyme. We used both approaches chronologically in this order.

Transgenic mice were first generated by introducing the *E. coli ada* gene attached to the Chinese hamster metallothionein I gene promoter into mouse zygotes (Matsukuma *et al.*, 1989). Liver extracts from these transgenic mice carry about three times the activity of normal mice and levels can be increased up to about eight times after treatment of zinc, due to the metal-responsive promoter element (Nakatsuru *et al.*, 1991). Groups of transgenic and non-transgenic mice were given an i.p. injection of $ZnSO_4$, and 10 hr thereafter were given an i.p. injection of either dimethylnitrosamine or diethylnitrosamine. There was a statistically significant reduction in liver tumor formation in transgenic mice of four of the six paired groups treated, and in the remaining two results were in line with dose dependence (Nakatsuru *et al.*, 1993). Transgenic mice expressing the *ada* chimeric gene or the human methyltransferase cDNA were also developed (Lim *et al.*, 1990; Fan *et al.*, 1990; Dumenco *et al.*, 1991) and, in this case, an apparent reduction in rate of induction of thymic lymphoma by MNU was observed (Dumenco *et al.*, 1993). Thus, methyltransferase can indeed protect animals from exposure to relatively low doses of alkylating carcinogenes.

In these transgenic mice, the suppressive effects on tumor formation were evident only when relatively high doses of alkylating agents were used. Moreover, as levels of expression of the transgenes vary with tissues, it can be difficult to define the protective effects of the enzyme. One would need to construct mice defective in their own methyltransferase genes. For this, elucidation of the genomic sequence for mouse methyltransferase is necessary, and so it was done (Shiraishi *et al.*, 1992; Iwakuma *et al.*, 1996).

Mouse lines deficient in the methyltransferase gene were established by gene targeting (Tsuzuki *et al.*, 1996). Tissues from these mice contained essentially no methyltransferase protein. Administration of MNU (50 mg/kg of body weight) to these gene-targeted mice led to early death whereas normal mice treated in the same manner showed no untoward effects. In methyltransferase-deficient mice given MNU treatment, the bone marrow became hypocellular and there was a drastic decrease in the number of leukocytes and platelets in peripheral blood, thereby indicating an impaired reproductive capacity of hematopoietic stem cells. Methyltransferase apparently protected these mice from the pancytopenia caused by the alkylating agent.

When varied doses of MNU were administered to 6-week-old mice and the survivors on the 30th day were counted, LD_{50} of $MGMT^{-/-}$ and $MGMT^{+/+}$ mice was 20 and 240 mg/kg of body weight, respectively (Fig. 3). Then, a sublethal dose of MNU (2.5 mg/kg of body weight, as shown by an arrow in the figure) was

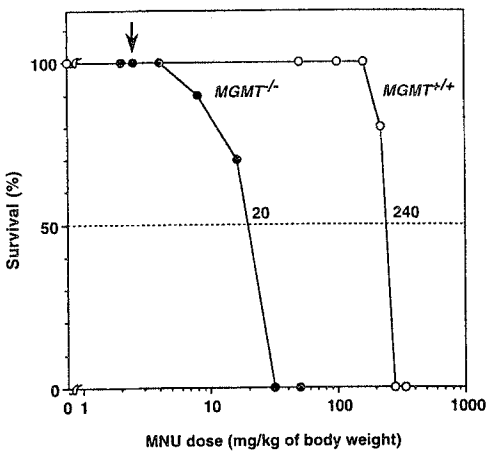

Fig. 3. Survival of $MGMT^{+/+}$ and $MGMT^{-/-}$ mice given different doses of MNU. Survival rates at the 30th day after MNU injection were plotted. Data were taken from Sakumi *et al.* (1997).

applied and the animals were observed for 28 weeks. Thymic lymphoma and lung adenoma were present in many of $MGMT^{-/-}$ mice while there was only one lung adenoma in the $MGMT^{+/+}$ mice (Sakumi *et al.*, 1997). In $MGMT^{-/-}$ female mice given an i.p. injection of dimethylnitrosamine (5 mg/kg), a significantly larger number of liver and lung tumors were produced, as compared with $MGMT^{+/+}$ mice treated in the same manner (Iwakuma *et al.*, 1997). Thus, it is clear that mice with a defect in the *MGMT* gene are indeed susceptible to alkylation-induced tumorigenesis.

It has been proposed that the accumulation of alkylated bases in chromosomal DNA may provoke abortive mismatch repair, thereby leading to cell death (Branch *et al.*, 1993; Kat *et al.*, 1993). It is now possible to test this hypothesis by constructing mice defective in both methyltransferase and mismatch repair genes. By making use of methyltransferase-deficient mice, one could also evaluate the extent of an endogenous alkylation-induced DNA lesion that would lead to induction of mutation as well as to cancer.

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