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The DNA Repair Protein O^6-Methylguanine-DNA Methyltransferase Protects against Skin Tumor Formation Induced by Antineoplastic Chloroethylnitrosourea[1]

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Abstract

Chloroethylnitrosoureas (CNUs) are being used in the therapy of various neoplastic diseases, including skin cancer. Because secondary tumor formation is a serious threat in chemotherapy with these drugs, we explored whether and to what extent the DNA repair protein DNA-O^6-methylguanine:protein-L-cysteine *S*-methyltransferase (MGMT) protects against CNU-induced tumors. We made use of transgenic mice overexpressing human MGMT in their skin and the initiation-promotion protocol on treatment with 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU, nimustine) that is representative of CNUs. ACNU applied topically as a single low dose to the dorsal skin was highly effective in tumor induction in nontransgenic mice, whereas in *cytokeratin MGMT* transgenic mice, tumor formation was remarkably reduced. ACNU-induced skin tumors harbored mutations in the c-Ha-*ras* gene in both groups of mice. The results provide clear evidence that MGMT exerts protection against CNU-induced cancer. Our data also indicate that O^6-chloroethylguanine, which is repaired by MGMT, is a main precarcinogenic CNU-induced DNA lesion.

Introduction

CNUs[3] represent an important class of chemotherapeutic drugs. They are in clinical use for treatment of various neoplastic diseases such as brain tumors, Hodgkin's disease, lymphoma, myeloma, and skin cancer (1). A paradox in the activity of these agents, like that of many other cytostatic drugs, is that they are not only antineoplastic but bear carcinogenic potency. In fact, secondary neoplasia brought about by therapeutic treatment of the primary tumor is a serious problem in high-dose cancer therapy (2, 3). Therefore, insight into the mechanisms protecting nontumor cells from the carcinogenic effect of these agents is highly desired.

Chloroethylating agents are strong alkylators, producing DNA monoadducts at various sites in DNA. A major alkylation site is the N^7 of guanine, in which the majority of base damage is produced. Less frequent attack occurs at the O^6 of guanine, giving rise to O^6-chloroethylguanine and O^6-hydroxyethylguanine (4). Furthermore, DNA interstrand cross-links are formed (4–6). These are considered to be the ultimate cytotoxic lesions responsible for the cytostatic effect and the myelosuppressive side effects of the agents. The formation of cross-links has been shown to result from chloroethylation at the O^6 position of guanine followed by intramolecular cyclization involving the N^1 of guanine. The resulting unstable intermediate (1,O^6-ethanoguanine) finally reacts with the N^3 position of cytosine on the opposite DNA strand, thus forming DNA interstrand cross-links that block DNA replication (5, 6).

CNUs were also shown to exert mutagenic effects in prokaryotic and eukaryotic cells (7, 8). Their carcinogenic potency is indicated by their ability to induce neoplastic transformation in rodent bioassays (9) and the occurrence of secondary malignancies after CNU chemotherapy (2, 3, 10). Although some data are available on the mutation spectrum of BCNU in mammalian cells treated in culture (11, 12), the major DNA lesions responsible for tumor formation on treatment with chloroethylating drugs are not yet defined.

O^6-Methylguanine as well as larger adducts involving chloroethylations at the O^6 position of guanine is removed by the DNA repair protein MGMT. Due to this repair reaction, the formation of N^1-guanine-N^3-cytosine cross-links is prevented (13), which results in a dramatic increase in survival of cells and suppression of recombination events on CNU treatment (14, 15). Interestingly, the cytotoxic and recombinogenic protection mediated by MGMT is agent specific (15), indicating that differences between CNUs might exist in the spectrum of genotoxic DNA lesions they induce. MGMT also protects against mutation induction on BCNU treatment (12), indicating that O^6-chloroethylguanine is not only a pretoxic but also a premutagenic lesion. It should be noted that MGMT reacts with 1,O^6-ethanoguanine in DNA too, therefore the repair protein can become covalently bond to DNA (16). The biological significance of the resulting protein-DNA cross-link is unknown.

Based on *in vivo* studies with *MGMT* transgenic mice, compelling evidence was provided that MGMT effectively protects from the carcinogenic effect of simple methylating agents, such as MNU (17, 18), which firmly established the decisive role of O^6-methylguanine as a main precarcinogenic lesion responsible for the development of different types of tumors. Using the two-stage skin carcinogenesis protocol, MGMT was shown to exert protection against tumor initiation without affecting the mechanism underlying tumor promotion (18).

Although the role of O^6-chloroethylguanine as a main pretoxic lesion induced by chemotherapeutic CNUs is well known, evidence is lacking that this lesion is also responsible for the carcinogenic effect of the agents. Moreover, there is no evidence to show that MGMT protects against CNU-induced carcinogenicity at all. We addressed this issue by using transgenic mice overexpressing the human MGMT protein in epidermal cells (18) and compared the tumor response after topical ACNU treatment of *CkMGMT* transgenic mice with that of normal mice expressing considerably lower amounts of MGMT in their skin. We made use of the initiation-promotion protocol, which is one of the best-characterized multistage carcinogenesis models. We

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[3] The abbreviations used are: CNU, chloroethylnitrosourea; ACNU, 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; MNU, *N*-methyl-*N*-nitrosourea; MGMT, DNA-6-*O*-methylguanine:protein-L-cysteine *S*-methyltransferase (EC 2.1.1.63); Ck, cytokeratin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; nt, nucleotide.

have chosen ACNU for the initiating treatment because this drug is a representative of CNUs that yield electrophilic species that chloroethylate DNA. Previously it was shown that MGMT exerts strong protection against the killing and recombinogenic activity of ACNU (15). Here we show that skin-targeted MGMT overexpression markedly reduces ACNU-induced skin tumor formation. Furthermore, we show that ACNU induces mutations in the c-Ha-*ras* gene. This occurred with similar frequency and mutational specificity in tumors that appeared on the skin of transgenic and nontransgenic individuals, suggesting that the same kind of nonrepaired DNA lesions give rise to tumor formation in both groups.

Materials and Methods

Transgenic Mice. The generation of transgenic mice harboring human *MGMT* cDNA under the control of *Ck* promoter elements has been described previously (18). *MGMT* transgene expression was targeted to the interfollicular epidermis and the outer cells of the hair follicles by virtue of the bovine *Ck III* (BK5) and *IV* (BK6β) promoter. The total cellular methyltransferase activity was shown to be significantly higher in transgenic as compared to nontransgenic skin. The transgenic Gat:NMRI mouse line *CkMGMT#3* was maintained in our laboratory as a homozygous colony with respect to the transgene.

Two-Stage Skin Carcinogenesis Experiments. ACNU (nimustine; Asta Medica) was dissolved in methanol (50 μmol/ml) and used immediately. Nine-to-twelve-week-old *CkMGMT* transgenic (n = 25) and nontransgenic Gat:NMRI mice (n = 27) were initiated by the application of 5 μmol of ACNU in 100 μl of methanol to the shaved dorsal skin. At day 7 postinitiation, tumor promotion was achieved by topical treatment with 10 nmol of TPA (Sigma) in 100 μl of acetone/animal twice weekly for 26 weeks. Noninitiated Gat:NMRI controls (n = 21) were treated with 100 μl of methanol 1 week before TPA-mediated promotion was carried out as described. Mice were examined weekly for the appearance of papillomas. The tumor response was determined per individual and expressed as tumor rate (number of papilloma-bearing mice/number of survivors) and tumor yield (number of papillomas/number of survivors). During this period, regrowing hairs were kept short by scissors.

Isolation of DNA. Papillomas were harvested from the back skin of all tumor-bearing mice. Genomic DNA was isolated from individual papillomas using either the phenol-chloroform extraction method (19) or a QIAamp tissue kit (Qiagen).

Amplification of Exons 1 and 2 of c-Ha-*ras* by PCR. Oligonucleotide primers used for PCR amplification of exon 1 of c-Ha-*ras* were P28 (5′-CTTGGCTAAGTGTGCTTCTCATTGG-3′; nt −69 to −45) and P29 (5′-CACCTCTGGCAGGTAGGCAGAGC-3′; nt 137–111), and primers for amplifying exon 2 were P36 (5′-CCTCCTCAGACCAGAGAATCC-3′; nt 269–247) and P42 (5′-GTGTTGTTGATGGCAAATACACAGAGG-3′; nt 467–441). The given nt numbers correspond to the mouse c-Ha-*ras* sequence published by Brown *et al.* (20). A total of 0.5–1.0 μg of genomic DNA was amplified in a total volume of 100 μl containing 0.5 μM each primer (P28/P29 or P36/P42), 200 μM deoxynucleotide triphosphates, 10 mM Tris-HCl (pH 8.3), 1 mM $MgCl_2$, and 1.5 units of Taq polymerase (Promega or Biomaster) using an M. J. Research minicycler (Biozym Diagnostik). After an initial denaturation at 94°C for 4 min, samples were subjected to 50 PCR cycles at 95°C, 59°C (for annealing of exon 1 primers) or 57°C (for annealing of exon 2 primers), and 72°C (1 min each), followed by a final extention at 72°C for 5 min.

Mutation Detection by RFLP and Sequence Analysis. To detect c-Ha-*ras* point mutations, PCR fragments were screened for RFLPs and subsequently sequenced. C-Ha-*ras*-activating mutations of the G→A transition type at the 2nd position of codon 12 create a new restriction site for *Eco* 57 I (CTGGAG→CTGAAG[N]$_{16/14}$). The same base substitution leads to the loss of a *Gsu*I recognition site (21). Mutations at position 2 or 1 of codon 13 can be distinguished by a loss of *Mnl*I cleavage and/or *Mwo*I restriction; a G→T transversion at position 2 produces a *Hin*fI restriction site (GAGGC→GAGTC; Ref. 22). The PCR products were purified using a QIAquick PCR purification kit (Qiagen). They were digested by a 10-fold excess of the indicated restriction enzymes and electrophoretically separated on a 3% agarose gel. Selected PCR products were ligated into pBluescript II (pCR-Script SK cloning kit; Stratagene) and subsequently sequenced (ALF; Pharmacia).

Results

Protection against ACNU-induced Papillomas in *CkMGMT* Transgenic Mice. *CkMGMT* transgenic mice as well as nontransgenic Gat:NMRI mice were treated according to the well-established two-stage skin carcinogenesis protocol. For tumor initiation, a single dose of 5 μmol of ACNU/mouse was applied topically to the back skin before TPA-mediated promotion was performed. ACNU-treated Gat:NMRI mice developed the first papilloma at week 12, whereas in transgenic mice, the first papilloma appeared at week 17 after the beginning of TPA promotion. The mean latency period for papilloma development was 21.1 weeks in nontransgenic mice and 22.5 weeks in transgenic mice. Twenty-four weeks after ACNU initiation, the frequency of papilloma-bearing mice (tumor rate) was 29.6% in nontransgenic mice and 4.2% in CkMGMT mice. At the end of the experiment, 44.4% of nontransgenic and 12.5% of transgenic animals developed at least one tumor after ACNU initiation (Fig. 1*A*). The number of tumors (tumor yield) that appeared at week 24 after ACNU application in nontransgenic and transgenic mice was 0.33 and 0.04 papilloma/mouse, respectively, whereas at week 27, nontransgenic

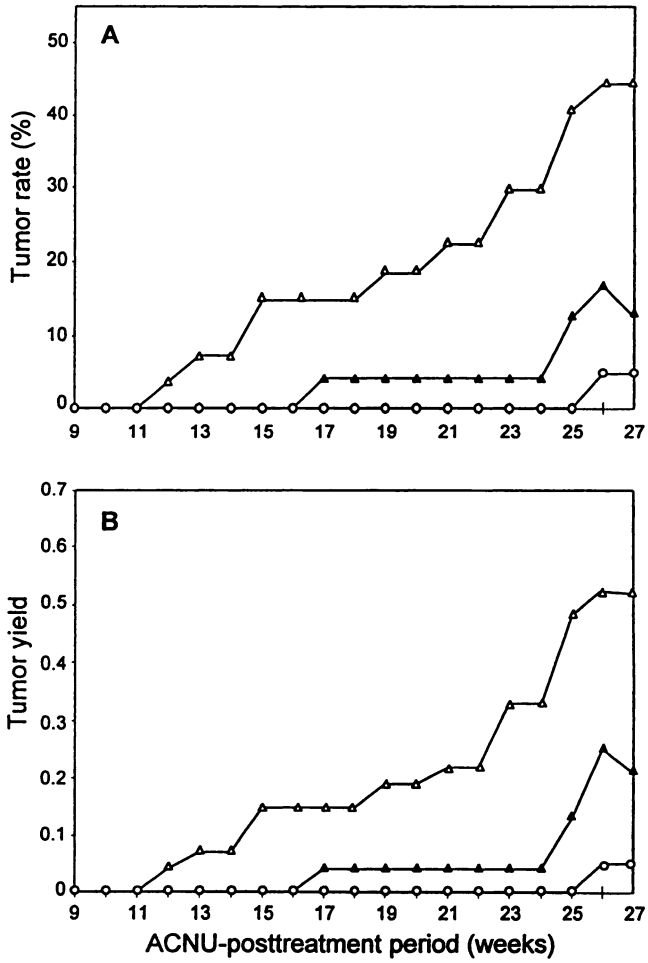

Fig. 1. Tumor response of *CkMGMT* transgenic and nontransgenic mice after ACNU initiation and TPA promotion. *CkMGMT* transgenic (▲) and nontransgenic mice (Δ) were topically treated with a single dose of 5 μmol of ACNU in 100 μl of methanol followed by 10 nmol of TPA (in 100 μl of acetone) twice weekly for 26 weeks. Noninitiated NMRI controls (○) were treated with 100 μl of methanol before TPA promotion. The tumor response was calculated weekly as tumor rate [*A*, (the number of papilloma-bearing mice/number of survivors) × 100] and tumor yield (*B*, the number of papillomas/number of survivors).

Table 1 *Incidence of base substitution mutations in codons 12, 13, and 61 of c-Ha-ras detected in ACNU- and MNU-induced papillomas of CkMGMT transgenic and nontransgenic mice*

Initiating agent	Mouse line	No. of papillomas analyzed	Base substitution mutations					
			Total	Codon 12 $G^{35}\rightarrow A$	Codon 13 $G^{38}\rightarrow A$	Codon 13 $G^{38}\rightarrow T$	Codon 13 $G^{38}C\rightarrow TT$	Codon 61[a] $A^{182}\rightarrow T$
ACNU	Nontransgenic Gat:NMRI	11	5/11 (45%)	2/5	0/5	1/5	2/5	0/4
ACNU	*CkMGMT* transgenic	5	2/5 (40%)	2/2	0/2	0/2	0/2	0/2
MNU	Nontransgenic Gat:NMRI	41	30/41 (73%)	29[b]/30	2[b]/30	0/30	0/30	0/11
MNU	*CkMGMT* transgenic	34	27/34 (79%)	27[c]/27	3[c]/27	0/27	0/27	0/6

[a] A → T mutations in codon 61 of c-Ha-*ras* were demonstrated to occur in papillomas of uninduced but TPA-treated mice (32) and represent the main DMBA-induced *ras* mutation in skin tumors (33, 34).
[b] Includes one double mutation $G^{35} \rightarrow A$, $G^{38} \rightarrow A$.
[c] Includes three double mutations $G^{35} \rightarrow A$, $G^{38} \rightarrow A$.

mice exhibited 0.52 papilloma/individual as compared to 0.21 papilloma/transgenic mouse (Fig. 1*B*). Thus, transgenic mice overexpressing MGMT in their skin were clearly protected from ACNU-induced skin tumor formation.

ACNU-Induced Oncogenic c-Ha-*ras* Mutations. C-Ha-*ras* is frequently activated in skin papillomas induced by methylating carcinogens, such as MNU (22). To find out whether or not ACNU-induced tumors also display mutations in c-Ha-*ras*, DNA samples prepared from papillomas that were harvested at the end of the experiment (week 27 after ACNU initiation) were analyzed for the presence of base substitutions in exons 1 and 2 of the gene. For this purpose, papilloma DNA was amplified by PCR, screened for RFLPs, and sequenced (Table 1). RFLP analysis of codon 12 was based on a newly created *Eco* 57 I restriction site suitable for detecting $G^{35}\rightarrow A^{35}$ point mutations. The DNA samples that gave the diagnostic fragments after *Eco* 57 I incubation were checked for a loss of *Gsu*I cleavage (21) and subsequently identified by sequencing as GGA→GAA mutations. Five of 11 papillomas (45%) of ACNU-treated nontransgenic mice harbored a mutation in either codon 12 or 13 of c-Ha-*ras*; only 2 of them were shown to be G→A transitions. Three papillomas contained a codon 13 mutation; one mutation was a G→T transversion at position 2, and two turned out to be GC→TT tandem base substitutions. Five papillomas were harvested from transgenic mice, and two of them showed G→A transitions involving the second base of codon 12 (see Table 1).

For comparison, papillomas obtained in two-stage skin carcinogenesis experiments after initiation by MNU (18)[4] were also screened for c-Ha-*ras* mutations by RFLP and sequence analysis (Table 1). The majority of papillomas (73 and 79% of control and transgenic mice, respectively) induced by MNU displayed base substitution mutations in the amplified exons of c-Ha-*ras* with predominating G→A transitions in codon 12 (97 and 100% of the mutations in nontransgenic and transgenic mice, respectively).

Discussion

There is clear evidence, based on the response of transgenic mice, that the repair protein MGMT protects against alkylation-induced carcinogenesis (17, 18, 23, 24). Moreover, using MGMT skin-targeted transgenic mice, we showed that the protective effect of MGMT pertains to the suppression of tumor initiation, whereas the mechanisms responsible for TPA-mediated tumor promotion remained unaffected (18). The experimental system used, *i.e.*, the initiation-promotion protocol on mouse skin, has the advantage that the initiator is applied as a rather low, subthreshold dose as compared to the doses to be used in complete carcinogenesis studies.

To investigate the role of MGMT in defense against the carcinogenic effect of clinically important CNUs and to define the major CNU-induced precarcinogenic DNA lesion(s), we treated transgenic mice overexpressing human MGMT in their skin together with nontransgenic controls with a single dose of ACNU followed by TPA promotion. The susceptibility of *CkMGMT* transgenic mice to the papilloma-inducing effect of ACNU was clearly reduced as compared to that of nontransgenic mice treated in the same way. The protecting effect exerted by MGMT was remarkable; tumors seemed delayed in *CkMGMT* mice, and the tumor frequency was significantly lower than that in the nontransgenic control group. There was an approximately 7-fold reduction of the tumor rate (percentage of tumor-bearing mice) at week 24 and a 4-fold reduction at the end of the promotion period at week 27.

Thus far, the tumor-preventing effect of MGMT *in vivo* was only shown for simple methylating agents, such as MNU and dimethylnitrosamine (17, 18, 23). For these agents, it is well known that O^6-methylguanine is the major premutagenic and precarcinogenic DNA lesion. Therefore, it was not surprising that the enhanced removal of this lesion from DNA reduced the tumor incidence. For CNUs, however, which represent a major class of antineoplastic agents, the critical precarcinogenic lesions are not known. It is accepted, however, that MGMT removes only monoadducts, such as chloroethyl groups, from the O^6 position of guanine. Therefore, it is reasonable to conclude from our experiments that O^6-chloroethylguanine is the major precarcinogenic primary lesion induced by ACNU and other CNU derivatives. This is especially remarkable in the light of the quantitative distribution of CNU-induced DNA modifications. About 95% of all guanine-targeted monoadducts were shown to be N^7-guanine alkylations, and only 2–3% were identified as O^6-guanine alkylations (25, 26). It should be noted that this minor lesion is mainly responsible for the cytotoxic and recombinogenic effect of ACNU (15). Obviously, the same lesion that is required to kill tumor cells may initiate tumor formation in normal cells, provided they survive the exposure to the alkylating agent. It would be interesting to see whether the secondary-formed guanine-cytosine cross-link gives rise to tumor formation or whether tumor initiation is due to the replication of DNA at sites harboring the primary lesion O^6-chloroethylguanine or the intermediary reaction product. It should be noted that hydroxyethylations can also be formed at the O^6 position of guanine. This adduct is also removed by MGMT, albeit at low efficiency (13). Therefore, it cannot be excluded that O^6-hydroxyethylguanine may also play a role in CNU-induced tumor initiation.

To define mutations responsible for the development of papillomas after ACNU treatment, codons 12 and 13 of the c-Ha-*ras* gene were screened for base substitutions. Both codons of the *ras* gene were shown to be the main target for oncogene activation by methylating agents in different kinds of tumors in rodents (27). We showed that 5 of 11 ACNU-induced papillomas of nontransgenic mice and 2 of 5 papillomas of transgenic mice harbored mutations in codons 12 and 13 of c-Ha-*ras* exon 1. For comparison, the incidence of c-Ha-*ras* mutations in papillomas induced by MNU was 73% (30 of 41) in nontransgenic mice

[4] K. Becker, unpublished observations.

and 79% (26 of 33) in *CkMGMT* transgenic mice. The spectrum of *ras* oncogene mutations induced by either ACNU or MNU comprised almost exclusively G-targeted single-base mutations, mainly G→A transitions. This is consistent with the assumption that persisting O^6-alkylguanine lesions are the main cause of ACNU- and MNU-induced carcinogenesis. Interestingly, the mutation spectrum, after treatment with both agents, did not differ between MGMT-overexpressing and nontransgenic mice. This suggests that the MGMT repair capacity in transgenic individuals was saturated by the given amount of DNA alkylations produced by these agents and that the same lesions gave rise to tumor formation in transgenic and nontransgenic individuals. The G→T transversion and GC→TT tandem base substitutions found after ACNU exposure were not observed after MNU treatment. This is in line with previous *in vitro* studies. Thus, in cultivated MGMT-deficient cells, the mutational specificity of the CNU BCNU was dominated by G→T transversions and G→A transitions. Occasionally, some G-targeted tandem transversions were also found (11). Mutational fingerprints after CCNU treatment revealed that more than 90% of the mutations were G-targeted base substitutions, mainly G→A transitions (28, 29). This emphasizes the significance of O^6 alkylation of guanine also for BCNU- and CCNU-mediated mutagenesis.

In summary, the data provide compelling evidence that MGMT protects against tumor formation brought about by antineoplastic treatment with ACNU. Because the drug is representative for CNUs with regard to the induced DNA damage, the protection against tumor formation mediated by MGMT very likely pertains to many other CNUs used in tumor therapy, such as BCNU, CCNU, 1-(2-chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea, fotemustine, and chlorozotocin. Bearing in mind the finding that MGMT also prevents methylnitrosourea-induced tumor formation (17, 18), the tumorigenic defense of MGMT can be extended to O^6-methylguanine-generating cytostatic drugs such as procarbazine, dacarbazine, and streptozotocin. Secondary neoplasias are a serious problem in the therapy of childhood and adult tumors with CNUs and methylating drugs (2, 3, 9, 30). Therefore, there is a need to develop strategies aimed at enhancing the expression of MGMT in nontarget tissues. Thus, transfer of the *MGMT* gene in hematopoietic stem cells expressing very low amounts of MGMT (31) would be highly desired both to protect against the myelosuppressive toxicity of CNUs and methylating drugs and to prevent therapy-related secondary hematopoietic malignancies in cancer patients.

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