Amplification of the DNA Repair Gene O6-Methylguanine-DNA Methyltransferase Associated with Resistance to Alkylating Drugs in a Mammalian Cell Line*

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The cytotoxic action of such alkylating chemotherapeutic drugs as 2-chloroethyl-N-nitrosourea (CNU) derivatives is countered by the repair protein O6-methylguanine-DNA methyltransferase (MGMT), which removes O6-alkylguanine induced in the DNA by these agents. Resistance to these drugs is often correlated with the MGMT levels in normal and tumor cells of human and rodent origin. Exposure of mouse 3T3 cells to increasing concentrations of CNU, and subsequent selection of resistant cells, led to the isolation of clones with 5–10 times higher levels of MGMT activity than in the control. The increased MGMT expression at both mRNA and protein levels resulted from 5–10-fold amplification of the Mgmt gene. Amplification of this gene was not associated with concomitant amplification of another alkylating damage repair gene, N-methylpurine-DNA glycosylase. No amplification of at least three other genes on chromosome 7 (which contains the Mgmt gene) was observed in the drug-resistant cells. Furthermore, the amplified Mgmt sequence was not associated with a homogeneously staining region, or double minute chromosomes, nor present as episomal DNA. In situ hybridization of metaphase chromosomes of the drug-resistant cells indicated both translocation and localized amplification of the Mgmt gene.

Development of resistance to drugs in mammalian tumor cells is a general phenomenon and is the major cause of failure of cancer chemotherapy (1–3). Thus, an understanding of the molecular basis of development of drug resistance is extremely important for designing new approaches to counter the progression of this process. Commensurate with the diversity of cytotoxic agents and their cellular targets, drug resistance often involves increased expression of specific genes. In the case of the widely studied multiple drug resistance gene, Mdr-1, the encoded product acts as an effluxing pump and reduces the intracellular concentrations of many chemotherapeutic drugs (1–3).

Several alkylating agents are being extensively used as potent antitumor drugs. These include nitrogen mustards, procarbazine, and various derivatives of 2-chloroethyl N-nitrosourea (CNU)\(^1\) \(\text{\textendash}\) (4). The CNU-type drugs exert their cytotoxic effect by cross-linking DNA strands, and the first step in the reaction involves O6-alkylation of guanine. Procarbazine also induces O6-alkylguanine but not DNA cross-links (4). Additionally, these alkylating agents also induce a significant amount of various N-alkylpurine adducts in DNA (4, 5).

The O6-alkylguanine and N-alkylpurines are removed from DNA by two ubiquitous DNA repair proteins, O6-methylguanine-DNA methyltransferase (MGMT) and N-methylpurine-DNA glycosylase (MPG), respectively (6). MGMT removes O6-alkylguanine (and the less abundant O4-alkylthymine less efficiently) in a single-step stoichiometric reaction involving direct transfer of the alkyl group to the protein (7). This mechanism effects inactivation of MGMT and restoration of the original base (8). In contrast, repair of N-alkylpurines in DNA involves the multi-step base excision repair pathway initiated by the removal of the base adduct by MPG, followed by repair of the resulting abasic site (9). Thus MGMT, as the sole protein involved in removing O6-alkylguanine from DNA, is rate-limiting in its repair. Consistent with the rate-limiting activity of MGMT, several studies have established a direct correlation between the MGMT level in a given cell type and its resistance to CNU derivatives and procarbazine (6, 10, 11). Furthermore, many alkylating drug-resistant tumors and cell lines have higher levels of MGMT than their progenitor cells (6, 11). The effect of MGMT level on sensitivity to both CNU and such monofunctional alkylating agents as N-methyl N’-nitro-N'-nitrosoanilide indicated that O6-alkylguanine, even without subsequent interstrand cross-links, is cytotoxic (11, 12). Although N-alkylpurines have been shown to be lethal lesions in Escherichia coli (7), their contribution to the killing of mammalian cells by alkylating drugs has not been extensively studied. However, some CNU-resistant tumor cells appear to have low levels of MGMT and remove N-alkylpurines residues more efficiently from their DNA than do normal cells (5). In any event, these results show that resistance to alkylating drugs can often be due to increased levels of DNA repair proteins.

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\(\text{\textendash}\) The abbreviations used are: CNU, 2-chloroethyl-N-nitrosourea; DM, double minute; HSR, homogeneously staining region; Igf, insulin-like growth factor receptor; MGMT, O6-methylguanine-DNA methyltransferase; MPG, N-methylpurine-DNA glycosylase; PKC, protein kinase C; FITC, fluorescein isothiocyanate.
Gene amplification is frequently observed in tumors and may be associated with the chromosomal instability of tumor cells (13–17). Although gene amplification may not occur in normal mammalian cells, nontransformed rodent cell lines, presumably because of their inherent genomic instability, often contain variants with an increased copy number of selectable genes (18). While overexpression of the Mgmt gene in many CNU-resistant human tumor lines tested so far appears invariably to be due to elevated levels of transcription, it is important to ask whether amplification of the Mgmt gene itself may also contribute to increased resistance to alkylating agents. An earlier study that preceded the cloning of the Mgmt gene suggested that amplification of this DNA repair gene is not responsible for drug resistance in tumor cells (19). In another study, V79 Chinese hamster cells selected for resistance to a nitrosourea derivative were found to contain both increased levels of MGMT and double minute chromosomes, indicative of gene amplification. However, no amplification of the Mgmt gene was detected in these resistant cells (20). In this paper, we describe the first reported amplification of the Mgmt gene in a mouse cell line, in parallel with the increased resistance of the cells to an alkylating drug.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Plasmids**—The NIH 3T3 cell line (a gift of W. K. Yang, Biology Division, Oak Ridge National Laboratory) was sub-cloned and then grown in Dulbecco’s modified minimal essential medium supplemented with 10% heat inactivated fetal bovine serum (Life Technologies, Inc.) and gentamicin (100 µg/ml). The clone had an average chromosome number of 65 and was free of double minute chromosomes (DM). The human and mouse MGMT cDNA-containing plasmids and human MGP cDNA-containing plasmid were described earlier (21–23); Int2, Igfr, and PKCβ cDNA-containing plasmids were kindly provided by E. Rinchik. CNU, a gift from Dr. N. Lomax of NCI, was freshly dissolved in water immediately before each use and diluted in water to obtain the desired concentration.

**Nucleic Acid Blot Hybridization**—High molecular weight DNA was extracted from the 3T3 cells by the method of Sambrook et al. (24) and digested to completion with different restriction endonucleases for Southern blot analysis. After quantitation of DNA by the fluorometric procedure (25), 10-µg aliquots of DNA were electrophoresed in 0.8% agarose and transferred to nylon membranes (Zeta-probe, Bio-Rad) containing 2.2 M formamide and transferred to nylon membranes (Zeta-probe, Bio-Rad) for Southern blot analysis. After quantitation of DNA by the fluorometric procedure (24), 10-µg aliquots of DNA were electrophoresed in 0.8% agarose and transferred to nylon membranes (Zeta-probe, Bio-Rad) under alkaline conditions. Purified DNA inserts from various plasmids were labeled with [α-32P]dCTP by the random priming method (26). Blots were hybridized at 42 °C in 50% deionized formamide, 6 × SSC, 0.25% non-fat dry milk with the radiolabeled probe, and washed to final stringency with 1 × SSC at 60 °C for 30 min (23).

**PCR Northern transfer analysis, poly(A)+** RNA was extracted from proteinase K-digested cell lysates by two successive oligo(dt)-cellulose chromatography steps according to Badley et al. (27). Denatured poly(A)+ RNA (2 µg) was electrophoresed on 1.2% agarose containing 2.2 M formamide and transferred to a nylon membrane (Hybond-N, Amersham). The membrane filters were baked at 80 °C for 1 h, hybridized at 65 °C in 0.5 M potassium phosphate (pH 6.5), 0.7% SDS, 0.25% non-fat dry milk, and washed as described above.

**DNA Probe**—Recombinant DNA probes used in this study were pSS600 (22) for the mouse MGMT cDNA; pDG23 (21) for human MGP cDNA; hPKCβ-15-Eco (28) for PKCβ mapped to chromosome 7 (from ATCC).

**MGMT Assay**—The MGMT activity in parent and CNU-resistant cells was quantitated using poly(dG dC)·[8-H3H]dG as the substrate as described earlier (29). The level of the methyltransferase was expressed on a per cell basis.

**Karyotype Analysis**—Metaphase cell preparations were made by standard procedures and stained with 3% Giemsa. Twenty cells were analyzed for chromosome number and the presence of DM chromosomes. Fluorescence in situ hybridization was carried out as described by Pinkel et al. (30) with some modifications (31). Briefly, biotinylated DNA probe was produced by nick translating a mouse Mgmt genomic clone in a cosmid, combined with yeast tRNA and salmon sperm DNA, and finally precipitated by adjusting to 0.3 M in sodium acetate (pH 5.6) and adding 2 volumes of cold ethanol. After standing for 2 h and centrifugation (10,000 × g, 15 min), the DNA was resuspended in 50% formamide, 2 × SSC, 50 mM Na-phosphate (pH 7.0) and hybridized to the cytological preparations for 16 h in situ. After hybridization, the slides were washed three times for 5 min in 60% formamide, 2 × SSC (pH 7.0) at 37 °C, then twice for 5 min each with 2 × SSC, and finally for 5 min with 4 × SSC, 0.5% Tween 20 at room temperature. The slides were washed with 5% natural non-fat dry milk (Lucerne Ltd.) in 4 × SSC for 10 min at room temperature, then washed with 4 × SSC, 0.05% Tween 20. The biotinylated probe was detected using fluorescein isothiocyanate (FITC, 5 ng/µl) conjugated with avidin (Vector Laboratories) for 30 min at room temperature before three washes with 4 × SSC, 0.05% Tween 20 for 5 min. The signals were amplified by reincubation twice with biotinylated anti-avidin D antibodies (5 ng/µl) before another incubation with avidin D and FITC for 30 min at room temperature. Finally, the slides were dehydrated, dried in ethanol, mounted in 20 mM Tris-HCl (pH 8.0), 90% glycerol containing 2% of antifade (1,4-diazabicyclo-(2.2.2)-octane, DABCO), propidium iodide (0.5 µg/ml) and 0.02% sodium azide, and examined under a Leitz microscope equipped with FITC epifluorescence optics.

Mouse Mgmt genomic pSS600 clones were isolated from a cosm id library.2 The total DNA was nick translated with fluorescein-tagged dATP and other unemployed dNTPs.

**RESULTS**

**Isolation and Characterization of CNU-resistant 3T3 Cell Clones**—The relative resistance of cells to CNU-type drugs is invariably dependent on their MGMT levels (6, 11, 12). Because of their relatively high level of MGMT, NIH 3T3 cells are more resistant to CNU than MGMT-deficient cells, e.g. Chinese hamster ovary cells (32). In preliminary experiments on cell killing as a function of CNU concentration, we observed that 300 µM CNU killed more than 90% of the cells. We isolated CNU-resistant clones by repeatedly exposing 3T3 cells to increasing concentrations of CNU, starting with 300 µM. The survivors after each treatment were allowed to grow to near confluence. 2 to 3 million cells/100-mm dish in the late log phase were treated with the drug for 30 min at 37 °C in serum-free medium. After removal of the drug, complete medium was added to the dishes, which were transferred to the CO2 incubator. Severe cytotoxicity was apparent as sloughing off most of the cells within 48 h after drug treatment. The remaining cells (<5% of the initial population) were allowed to grow in fresh medium for about 2 weeks when the cell density reached near confluence (4–5 doublings). The cells were split 1:5, transferred to fresh dishes, grown overnight, treated again with 300 µM CNU, and the whole cycle was repeated once more. The surviving cells after growth and subculture were then treated with 400 µM CNU for two cycles, followed by two cycles of exposure to 500 µM. Finally, the cells were exposed to two cycles of 600 µM CNU and single colonies of surviving cells were isolated. Increasing the CNU concentration above 600 µM did not allow any surviving cells that would eventually form colonies. Significant toxicity to 600 µM was observed even among the cells that grow from cells surviving two treatments with that drug concentration. Although about a dozen colonies were formed per dish after treatment with 600 µM, we picked three colonies with the highest cell numbers for further study, and named these presumptive clones CR-1, CR-2, and CR-3. These cells were maintained by treatment with 300 µM CNU before each passage; they showed little toxic response to this drug treatment. These cells showed a small increase in chromosome number (65 ± 10) compared with wild type cells, and some acentric chromosome fragments were observed. However, there was no evidence for acentric DM chromosomes or homogeneously staining regions (HSR) in any of the cell lines (data not shown). Unless otherwise stated, the following experiments were performed with the CR-1 line; experiments with CR-2 showed similar results.

**Amplification of the Mgmt Gene**—Equal amounts of DNA (20

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2 S. Shioita, unpublished data.
Selective Amplification of the Mgmt Gene—We further investigated whether amplification of the Mgmt gene was associated with amplification of other selected genes on the same or different chromosomes. Resistance of cells to CNU was invariably correlated with the level of MGMT, the enzyme responsible for the removal of O6-alkylguanine. Ludlum (5) has also shown that an increased repair of N^-alkylpurine adducts in DNA was observed in some tumor cells resistant to BCNU. Furthermore, this resistance was associated with an increased level of MPG activity (6). Therefore, we tested whether the Mpg gene was coamplified with the Mgmt gene in the CR-1 line. Fig. 1B shows the absence of MPG gene amplification in this clone, which, based on densitometric analysis, showed about a 6-fold increase in the Mgmt gene copy number over that of the parent line. The lack of MPG amplification also served as a positive control for equal loading of DNA samples in the gel.

The HSR is a hallmark of gene amplification in a locus where hundreds of copies of the amplified genes are present within a chromosome (18, 33, 34). The lack of any HSR in metaphase chromosomes of CR-1 suggested the absence of such extensive amplification of Mgmt and other genes. The mouse Mgmt gene has been localized near the telomere of chromosome 7 at 24.3 centimorgan distal from the centromere, 8.8 centimorgan distal from PKC-β, and 4.6 centimorgan proximal from the Int2 locus (35). ③ Very few genes in the vicinity of the Mgmt gene have been cloned or identified (35). We asked whether Mgmt gene amplification affects Igfr, Int-2, and PKC-β. Among these, Int-2 is the closest to Mgmt, and Igfr is the farthest. The filter containing CR-1 DNA was thus hybridized with these probes. We did not detect amplification of any of these genes (Fig. 1C), and so concluded that the amplified region of chromosome 7 was localized in the immediate vicinity of Mgmt gene.

Wahl and co-workers (33, 36) have shown that amplification of the CAD gene in Chinese hamster ovary cells involved formation of submicroscopic episomal DNA containing this gene. We followed the protocol described by Carroll et al. (36) to test whether the Mgmt gene could be detected in such episomal DNA in the CR-1 line. Blot hybridization of gel-fractionated DNA corresponding to the expected size of episomal DNA did not show any Mgmt-specific sequences (data not shown).

Taken together, our results show that CNU-mediated Mgmt gene amplification is not associated with amplification of another key alkylation repair gene and that the size of the amplified region is less than 4.6 centimorgan surrounding the Mgmt locus.

Mgmt Transcript Levels in CNU-resistant Cells—The increased number of the Mgmt gene was expected to result in an increased level of the Mgmt transcript. The level of Mgmt mRNA was indeed elevated 5–6-fold in CR-1 cells versus the parental 3T3 line when equal amounts of total RNA were loaded onto the gel for Northern blot analysis (Fig. 2). In several experiments, a 5–10-fold higher level of MGMT mRNA was present in log-phase CR-1 cells than in log-phase parental cells.

MGMT Activity in CNU-resistant Cells—Because resistance to CNU is associated with increased MGMT protein levels, it was expected that CR-1 cells would have a higher level of

③ N. Jenkins, personal communication.
MGMT activity as a result of Mgmt gene amplification. Table I shows that MGMT activity was elevated about 6-fold over that in 3T3 cells. Furthermore, there was an approximately colinear relationship among the increase in Mgmt gene copies, the level of mRNA, and the activity in CR-1 cells when compared with the 3T3 cells.

Chromosomal Localization of Amplified Mgmt Gene Sequences—Finally, we investigated the chromosomal location of the amplified Mgmt gene sequences in CR-1 cells by fluorescent in situ hybridization. The mouse Mgmt gene has been mapped near the telomere of chromosome 7 (Fig. 3) (35). Although we did not identify the individual chromosomes in metaphase spreads of control 3T3 and CR-1 cells, it is evident that the Mgmt gene signal is located only at the terminal region of chromosomes in the control 3T3 cells (Fig. 3A). On the average, the 3T3 line appears to be triploid with 60–70 chromosomes, and most of the cells had three Mgmt gene-carrying chromosomes. However, in some cells, an additional centric fragment, indicative of deletion in that chromosome, also contained the Mgmt gene sequence (Fig. 3A). The average number of chromosomes in CR-1 cells was not significantly different from that in the parent line. However, the Mgmt gene in 90% of CR-1 cells was present in four locations, of which three were terminal and one interstitial (Fig. 3B). A comparison of the strength of the signals of the control and CR-1 cells indicates significant amplification of the Mgmt gene present in the interstitial region of a chromosome in CR-1 cells. In other late passage preparations of CR-1 cells, four to five Mgmt signals were present in many cells, often with two amplified signals located in the middle of the arms. The presence of the Mgmt gene in the interstitial region suggests translocation, although the breakpoint cannot be defined.

DISCUSSION

Gene amplification in mammalian cell lines was first observed in association with increased resistance to antimitobolites, when cultured cells were selected in the presence of increasing concentrations of the drugs. The intracellular levels of the proteins targeted by these chemicals were elevated to counter their cytotoxic effects. While multiple mechanisms can cause an increased concentration of target proteins, a simple one is to increase the gene dosage without requiring a change in the promoter activity for the specific gene. Amplification of dihydrofolate reductase and Mdr-1 genes in rodent cells can be readily demonstrated when these are adapted to cytotoxic levels of methotrexate and anthracyclin drugs, respectively (1, 3).

It is now evident that amplification is one manifestation of genomic instability (17, 18). Gene amplification may not occur in normal diploid mammalian cells possessing functional p53 protein that is involved in maintaining genomic stability (15, 17). Although cells permissive for gene amplification often have inactive p53 (17, 18), amplification of drug-resistance genes has been observed in cells with wild type p53, presumably because of inactivation of another component in the regulatory pathway downstream of the p53 protein (14). Thus, although the 3T3 line used in our studies appears to contain wild type p53, these aneuploid cells are permissive for gene amplification because of another defect in the DNA damage-responsive pathway that prevents cell proliferation (14).

Amplification of the Mgmt gene as observed in 3T3 cells in our study is consistent with the unequal exchange model (18, 37, 38). The interstitial location of additional copies of Mgmt gene (Fig. 3B) is consistent with this breakage-fusion-bridge model for gene amplification although other models cannot be excluded without further experiments (33, 34, 37). Whether amplification of the Mgmt gene contributes to its overexpression (often observed in tumor cells) has important implications for chemotherapy with CNU derivatives. Earlier attempts to test indirectly for Mgmt gene amplification were unsuccessful (19). It is possible that the method based on reassocation kinetics used in that study was not sensitive enough for detecting the small increase in copy number of the Mgmt gene. In contrast to the situation with amplification of dihydrofolate reductase and other drug resistance genes where the number of gene copies per cell can be serially increased to a thousand or more by successive treatments with increasing concentrations of the selecting drug (14, 33), we observed that 3T3 cells could not be made more resistant by increasing the CNU concentration, and significant toxicity was observed even at 600 μM CNU in the cells that were originally selected in the presence of 600 μM CNU. A maximum of eight to ten Mgmt gene copies were present in these cells. Limited amplification of the Mgmt gene may be explained by the nature of the selection for cells containing amplified Mgmt. Unlike most other cases, where the target of the selecting drug is the gene product itself, the target of CNU is not MGMT itself, but there is an indirect relationship between the protein and the drug via a DNA lesion. CNU induces a number of cytotoxic lesions, of which O6-alkylguanine is the most important, and its effects can be countered by an increased level of MGMT. However, with increasing alkyl-

| Cell line | No. of MGMT molecules/cell | -Fold increase | Ratio of mRNA MGMT/γ-actin | -Fold increase |
|-----------|---------------------------|----------------|---------------------------|----------------|
| Control   | 41,600 (1.0)              | 0.04           | 1.0                       | (1.0)          |
| CR-1      | 273,800 6.6               | 0.43           | 10.8                      |
| CR-2      | 310,000 7.4               | 0.37           | 8.1                       |

FIG. 3. Fluorescent in situ hybridization of amplified Mgmt gene sequence in metaphase chromosomes of CNU-resistant clones of 3T3 cells. A, control 3T3 cells. B, CR-1 cells. Light arrows in panels A and B indicate locations of unamplified Mgmt genomic sequences. The heavy arrow in panel B indicates interstitial amplified Mgmt gene sequences.
tion, the contributions of other cytotoxic adducts (e.g., 7-alkylguanine) become significant, and these cannot be countered by MGMT (6). In principle, coamplification of genes encoding the repair proteins for the other adducts would be extremely rare.

and coamplification of more than one gene (on different chromosomes) would be extremely rare. Consequently, presumably because amplification is a rare event (18, 33) and coamplification of more than one gene (on different chromosomes) would be extremely rare.

In conclusion, this study provides the first evidence for amplification of a DNA repair gene. Although amplification of the Mgmt gene has not been observed in the relatively few human drug-resistant tumor lines tested so far, there is no a priori reason to assume that such does not occur, at least in some tumors surviving alkylating drug treatment.

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