Expression of Human O\textsuperscript{6}-Methylguanine-DNA Methyltransferase in a DNA Excision Repair-deficient Chinese Hamster Ovary Cell Line and Its Response to Certain Alkylating Agents\textsuperscript{1}

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ABSTRACT

A plasmid has been constructed in which the expression of human O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT) complementary DNA is driven by the Rous sarcoma virus promoter sequence. We had previously shown that transfection of this plasmid into Chinese hamster ovary (CHO) cells results in the expression of MGMT and in increased cellular resistance to N-methyl-N\textsuperscript{'},N\textsuperscript{'}-nitro-N-nitrosoguanidine and 1-(2-chloroethyl)-1-nitrosourea (CNU) but not N-nitroso-N-ethyletherua (ENU). In the present study, the Rous sarcoma virus promoter-MGMT was transfected into DNA excision repair-deficient CHO UV41 cells to investigate the phenotype associated with MGMT expression in the absence of DNA excision repair. Both the UV41/MGMT and CHO/MGMT cells expressed similar levels of MGMT and exhibited a similar increased resistance to N-methyl-N\textsuperscript{'},N\textsuperscript{'}-nitro-N-nitrosoguanidine. The UV41 cells were 20-fold more sensitive to CNU than the wild-type CHO cells. Expression of MGMT increased the resistance to CNU about 6-fold in both cell lines, but the difference between the two cell lines attributable to the excision repair defect still persisted. The UV41 cells were 2- to 3-fold more sensitive than the wild-type CHO cells to the monofunctional alkylating agents 1-(2-hydroxyethyl)-1-nitrosourea and ENU, but the MGMT phenotype did not alter sensitivity. This suggests that alkylation at the O\textsuperscript{6} position of guanine has no role in cytotoxicity of ethylating agents and that monofunctional DNA damage has little role in the cytotoxicity of CNU. Since MGMT can prevent the formation of G-C interstrand cross-links formed by CNU, other excision repair-sensitive DNA adducts must play a major role in the sensitivity of UV41 cells to this bifunctional alkylating agent. These results suggest that DNA intrastand cross-links may be major contributors to the cytotoxicity of CNU.

INTRODUCTION

CNUs\textsuperscript{3} are highly active agents that are used to treat a variety of tumors (1, 2). The CNUs produce both monofunctional and bifunctional alkylation of DNA. The cytotoxic effects of the CNUs are related predominantly to the formation of bifunctional adducts as hydroxyethyl-nitrosoureas, which produce only monofunctional adducts, are much less cytotoxic. The bifunctional adducts produced by CNU can be either DNA-protein, DNA-intrastrand, or DNA-interstrand cross-links, although it is generally believed that the latter cross-link represents the major cytotoxic lesion (3, 4). The formation of interstrand cross-links by the CNUs is a multistep process with the first step involving alkylation at the O\textsuperscript{6} position of guanine (5). This is followed by an intramolecular cyclization to form the unstable intermediate N',O\textsuperscript{6}-ethanoguanine. The latter intermediate interacts with the N\textsuperscript{7} position of a cytosine located in the opposite DNA strand, leading to the formation of an interstrand cross-link. It is important to note that the formation of this G-C cross-link is a relatively slow process. Both intermediates in the formation of this cross-link are substrates for the DNA repair protein MGMT (reviewed in Ref. 6). MGMT can prevent the formation of the DNA interstrand cross-link by cleaving the C-O bond and transferring the alkyl group to a cysteine residue, resulting in a S-alkylcysteine in MGMT. This transfer has been referred to as a "suicide" reaction, since the methyltransferase itself is inactivated (7). In the case of the N',O\textsuperscript{6}-ethanoguanine intermediate, a DNA-protein cross-link is formed. Proof for this latter pathway was obtained by demonstrating the binding of MGMT to CNU-treated DNA (8).

MGMT is an important protein involved in promoting cellular resistance to the cytotoxic effects of the nitrosoureas. Depletion of MGMT in resistant cells is accompanied by an increase in their sensitivity to chloroethylating agents (9). M\textsuperscript{ex} mammalian cell lines (i.e., cells not expressing MGMT) that are transfected with plasmid vectors which code for the Escherichia coli methyltransferase protein exhibit an increased resistance to the CNUs (10, 11). However, some evidence exists which suggests a role for other DNA repair mechanisms in cellular resistance to the CNUs (for review, see Ref. 12).

Recently, the successful cloning of the human MGMT cDNA was reported by three different groups (13—15). Furthermore, Hayakawa et al. (14) have reported that the expression of transfected human MGMT in Mex\textsuperscript{ex} human cells elicited resistance to the cytotoxic effects of MNNG and CNU. Our laboratory has been interested in the relative roles of MGMT and DNA excision repair systems in cellular resistance to alkylating agents. We previously constructed a human MGMT expression vector pRSV-MGMT, which was transfected into the excision repair-proficient wild-type CHO cells (16). Expression of human MGMT in the wild-type CHO cells protected the cells against the cytotoxic effects of MNNG and CNU. We now report on the expression of MGMT in an excision repair-deficient CHO cell line and the responses of these transfected cells to certain alkylating agents.

MATERIALS AND METHODS

CNU was obtained from Dr. Ven L. Narayanan of the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD, MNNG and ENU were purchased from Sigma Chemical Co. (St. Louis, MO). HENU was kindly provided by Dr. Sidney Mirvish of the Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha, NE.

All CHO cells were maintained as described (16). The CHO/UV41 cells which are an excision repair-deficient mutant of CHO/AA8 cells were obtained from Dr. Larry H. Thompson, Livermore, CA. The sensitivity of CHO cells to different alkylating agents was determined as the percentage of surviving cells by a colony formation assay (17). CNU, ENU, and HENU were dissolved in 100% dry ethanol immedi-

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3 The abbreviations used are: CNU, 1-(2-chloroethyl)-1-nitrosourea; ENU, N-nitroso-N-ethyletherua; HENU, 1-(2-hydroxyethyl)-1-nitrosourea; MGMT, O\textsuperscript{6}-methylguanine-DNA methyltransferase; RSV, Rous sarcoma virus; gst, xanthine guanine phosphoribosyltransferase; MNNG, N-methyl-N\textsuperscript{'},N\textsuperscript{'}-nitro-N-nitrosoguanidine; cDNA, complementary DNA; CHO, Chinese hamster ovary.

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ately before use, and MNNG was prepared as a 2 mM stock solution in 100% dry ethanol and stored at -20°C.

Plasmid pRSV-MGMT was constructed as described in a recent publication from this laboratory (16). The cDNA for human MGMT was amplified by a polymerase chain reaction procedure from HeLa cell mRNA and cloned into an expression vector. As a result, the human MGMT cDNA was flanked by a RSV-LTR promoter sequence and a polyadenylation site on the 5′- and 3′-ends, respectively. This plasmid pRSV-MGMT, was transfected into CHO cells as described previously (16). In brief, pRSV-MGMT (20 μg) and pSV2-gpt (2 μg used as a marker gene) were cotransfected with salmon sperm DNA (10 μg) onto the CHO cells by the calcium phosphate precipitation technique as practiced in our laboratory (17). Colonies that were positive for the bacterial gpt gene appeared in about 10 days. These were subcloned and cultured in selection medium (17) for another 10 days. Regular growth medium was used subsequently. MGMT-positive colonies from the latter were then selected. MGMT activity was assayed as described (18) by the transfer of the 3H-methyl group from methylated DNA to protein.

The sensitivity of CHO cells to alkylating agents. Cells were treated with MNNG (A), CNU (B), HENU (C), and ENU (D) for 1 h at 37°C. The percentage of survival was determined as described in “Materials and Methods.” Each point represents the mean of 3 determinations in a single experiment. Wild-type CHO (Δ), UV41 (○), CHO/A301 (●), UV41/303 (▲), and UV41/305 (△).

RESULTS

Endogenous MGMT activity is undetectable in extracts from both wild-type CHO cells and excision repair-deficient UV41 cells. Consequently, their phenotype is characterized as Mex- (19). The plasmid pRSV-MGMT (Fig. 1) was transfected into the cell lines together with a marker plasmid, pSV2-gpt. The resulting gpt+ cells were cloned and tested for MGMT activity. The MGMT activities of these clones were calculated from the mean of at least 3 points within the linear region of a protein-dependent assay. The introduction of pSV2-gpt alone into either cell line did not result in any increase in MGMT activity or resistance to alkylating agents (data not shown). CHO/MGMT clone A301, a clone that resulted from the transfection of wild-type CHO cells with pRSV-MGMT (16), was used in survival studies to compare with UV41/MGMT clones UV41/303 and UV41/305 which were derived from the transfection of UV41 cells with pRSV-MGMT. The cell-free extracts from CHO/A301, UV41/303, and UV41/305 cells had MGMT-specific activities of 0.30, 0.31, and 0.22 pmol/mg of protein, respectively.

The sensitivity of these cells to MNNG, CNU, ENU, and HENU was determined (Fig. 2). The UV41 cells were only slightly more sensitive to MNNG than the wild-type CHO cells, whereas the CHO/MGMT clone and both UV41/MGMT clones showed a significant resistance to the monofunctional alkylating agent, MNNG. This resistance to MNNG correlated well with the MGMT activity in these cells.

A much different picture was observed with CNU as the alkylating agent (Fig. 2B). The UV41 cells were 20-fold more sensitive to the cytotoxic action of CNU than the wild-type CHO cells upon comparison of the concentrations required to achieve 37% survival (D37). CHO/A301 cells were very refractory to the cytotoxic effects of this bifunctional alkylating agent with a concentration greater than 250 μM required to achieve D37. In contrast, UV41/303 and UV41/305 cells exhibited about 6-fold resistance to CNU compared with UV41 cells, but were still >25-fold more sensitive than the CHO/A301 cells despite their comparable levels of MGMT. Therefore, conversion to the MGMT phenotype produced approximately the same fold increase in resistance to CNU, but the original difference in sensitivity between the cell lines still persisted.

The possible contribution of monofunctional adducts to the sensitivity of UV41 cells was investigated using HENU which produces only this type of lesion. The UV41 cells were about 3-fold more sensitive to HENU compared with CHO cells, but the MGMT phenotype had no effect on HENU cytotoxicity in any of the cell lines (Fig. 2C). Similar results were obtained with ENU (Fig. 2D); although the UV41 cells were about 2-fold more sensitive than the wild-type CHO cells, the MGMT phenotype did not alter sensitivity.

Fig. 1. Map of the expression vector pRSV-MGMT. The MGMT cDNA sequences (MGMT cDNA), the remaining 3′ portion of the chloramphenicol acetyltransferase gene (3′-cat), the region of the RSV long terminal repeat (RSV LTR), the polyadenylation signal (AAA), ampicillin resistance gene (ampR), and the origin of replication (ori) are indicated.
DISCUSSION

DNA-damaging agents each produce a variety of DNA adducts that have variable potency with respect to cytotoxicity and mutagenicity. Accordingly, O\(^{\text{\prime}}\)-methylguanine is usually considered the most cytotoxic lesion produced by MNNG, even though it represents less than 10% of the total DNA adducts. In the case of CNU, a DNA-interstrand cross-link is considered the most cytotoxic lesion. This cross-link probably represents no more than 2% of the total DNA lesions. It is difficult to confirm the relative significance of each of these lesions, because they are always present with a high frequency of other adducts. The availability of cloned DNA repair genes can facilitate such studies by removing specific lesions so that any alteration in phenotype can be assessed.

We previously developed a series of CHO/MGMT cell lines and demonstrated that the sensitivity to MNNG correlated well with the level of MGMT activity (16). CHO/A301 was the clone in those studies that expressed the highest level of MGMT. The current experiments were designed to determine the phenotype that results when MGMT is expressed in an excision repair-deficient cell line. The UV41/MGMT cell lines developed here have levels of MGMT comparable to CHO/A301; the latter therefore provides a good comparison for the current experiments. The resistance of UV41/MGMT cells to MNNG also correlated well with the level of MGMT activity. These results suggest that the excision repair pathway which is deficient in UV41 cells contributes little to protecting cells from the cytotoxicity of MNNG. It has previously been shown that the DNA excision repair mechanism can repair O\(^{\text{\prime}}\)-methylguanine lesions (20), but this appears to have a minimal effect compared with the large increase in survival attributable to the expression of MGMT.

These results do not show that O\(^{\text{\prime}}\)-methylguanine is the only cytotoxic lesion nor even that it is the most cytotoxic lesion. Other cell lines exist that are hypersensitive to alkylating agents, such as MNNG, while retaining normal DNA excision repair capacity. CHO/EM9 is one such cell line that exhibits markedly increased sister chromatid exchange and a deficiency in repair of DNA strand breaks (21). The DNA breaks arise indirectly from the treatment with MNNG, presumably as a result of spontaneous or glycosylase-dependent depurination at alkylated purines followed by cleavage at the apurinic site. It remains to be determined what effect MGMT will have on the phenotype of such a cell line.

In contrast to MNNG, CNU-induced cytotoxicity was significantly different in wild-type CHO and UV41 cells. This is consistent with the observation that the UV41 cells are hypersensitive (30- to 90-fold) to bifunctional alkylating agents, such as mitomycin C, diepoxybutane, and cisplatin (22). It has been believed generally that this hypersensitivity is due to the inability to repair DNA interstrand cross-links. However, these cells are also unable to repair DNA intrastrand cross-links (23) which confounds a determination of the more critical lesion. If the DNA-interstrand cross-links are the major lethal lesions in UV41 cells, then preventing their formation by expressing MGMT should protect against the cytotoxic effect of CNU. Although introduction of MGMT into UV41 cells did increase resistance to CNU, these transformants were still at least 25-fold more sensitive to CNU than CHO/MGMT. Therefore, the difference between the CHO and UV41 cells was independent of the expression of MGMT. Accordingly, other lesions must contribute significantly to the sensitivity of UV41 cells to CNU, presumably lesions that are readily repaired by excision repair.

The possible contribution of monofunctional nitrosourea lesions to the sensitivity of UV41 cells was ruled out by testing the sensitivity of cells to HENU as well as ENU. The UV41 cells were about 2- to 3-fold more sensitive to both these agents, but expression of MGMT did not alter sensitivity. This suggests that monofunctional lesions cannot explain the majority of the difference between CHO and UV41 cells. In addition, this suggests that alkylation at O\(^{\text{\prime}}\)-guanine is insignificant with respect to the toxicity of these two ethylating agents, even though MGMT can repair these lesions in vitro (24). This leaves the question as to what bifunctional lesions are responsible for the cytotoxicity of CNU in the absence of excision repair. A likely candidate would be the 1,2-bis(7-deoxyguanosinyl)ethane cross-link (25). The covalent bond between two guanines could theoretically result from either cross-linking of two neighboring guanines on the same strand, i.e., intrastrand cross-link, or two guanines on each of the two DNA strands, i.e., interstrand cross-link. These cross-links, or any intermediate in their formation, are not substrates for MGMT. However, in the presence of MGMT, few if any interstrand cross-links are produced, suggesting that MGMT can protect against the formation of all such cross-links (26). Therefore, the 1,2-bis(7-deoxyguanosinyl)ethane bond would appear to represent the DNA intrastrand cross-link. Probably, other types of intrastrand cross-links also exist. A role of DNA excision repair in resistance to the CNUs has been suggested in mammalian cells. Bodell et al. (27) compared the qualitative and quantitative spectrum of modified bases in the DNA of sensitive and resistant CNU-exposed human tumor cells. Several significant differences in the distribution of specific lesions existed between the sensitive and resistant cells. However, only the dG-dC cross-link was repaired by the increased activity of MGMT, suggesting that the other differences resulted from other repair systems which contributed to the development of resistance to the CNUs.

The conclusion of these studies is that the sensitivity of UV41 cells to CNU is primarily due to their inability to repair intrastrand cross-links. This sensitivity can be overcome only partially by the expression of MGMT. Previous research has focused on MGMT as a critical determinant in the response of patients to CNU therapy. Our results emphasize the significance of DNA intrastrand cross-links and excision repair as alternate determinants of response.

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