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EFFECT OF ALKYL-*N*-PURINE DNA GLYCOSYLASE OVEREXPRESSION ON CELLULAR RESISTANCE TO BIFUNCTIONAL ALKYLATING AGENTS

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Abstract—Increased activity of alkyl-*N*-purine DNA glycosylase (ANPG; a.k.a. N^3-methyladenine DNA glycosylase) has been correlated with resistance to both chloroethylnitrosoureas and nitrogen mustards. Also, overexpression of the human glycosylase in *Escherichia coli* results in resistance to alkylating agents. To determine how overexpression of the protein affects resistance to these bifunctional alkylating agents in mammalian cells, wild-type CHO-AA8 cells were transfected with an expression construct containing the human ANPG cDNA. Several clonally isolated lines that expressed increasing levels of glycosylase activity were selected. None of these lines displayed increased resistance to either *bis*-chloroethylnitrosourea or melphalan. To determine how overexpression of this protein affects cells in the absence of nucleotide excision repair, the mutant CHO-UV20 cell line was transfected with the same expression construct. This cell line lacks functional ERCC-1 protein and displays extreme hypersensitivity to bifunctional alkylating agents. Again, none of the UV20 transfectants displayed increased resistance. The results of these experiments indicate that unlike *E. coli*, overexpression of the glycosylase alone is not sufficient to confer resistance to bifunctional alkylating agents in this system. Structural differences between mammalian cells and *E. coli* may explain the interesting result that a mammalian gene can confer drug resistance in *E. coli* but not in mammalian cells.

Key words: N^3-methyladenine DNA glycosylase; melphalan; carmustine; DNA repair

CNUs§ and NMs are among the most commonly used chemotherapeutic drugs in the treatment of a wide variety of tumors. The limiting factor in clinical use of these agents is tumor resistance, either innate or acquired. Toxicity of these drugs has been correlated to their ability to form cross-links in DNA, and resistance has been associated with enhanced repair of alkylation damage. Such repair is displayed through either reduced cross-link formation or enhanced cross-link removal. Classically, most DNA alkylation is repaired by one of two pathways: (1) BER or (2) NER. Both pathways have been implicated in resistance to CNUs and NMs [1–3]. The complexity of NER makes it difficult to identify key genes for alkylating agent resistance [4, 5]. BER involves fewer enzymes and therefore has been easier to characterize [6]. Repair begins with excision of the alkylated base by ANPG (a.k.a. N^3-methyladenine DNA glycosylase), resulting in the formation of an abasic site. The site is then cleaved by the sequential action of a 3′ exonuclease and a 5′ deoxyribosephosphodiesterase, which remove the depurinated sugar residue. The process is completed by "gap-filling" involving a polymerase and ligase.

The ANPG enzyme recognizes a wide variety of alkylated bases including N^3-methyladenine, N^3-methylguanine and N^7-methylguanine [7–9]. This enzyme is also capable of recognizing ethyl lesions [7, 8]. Thus, it is conceivable that this enzyme could recognize the N^7 lesions incurred by NM and CNU alkylation. A mutant CHO cell line that is hypersensitive to NM (6.8-fold) also displays hypersensitivity to MMS (10-fold), indicating that NM hypersensitivity may result from a defect in BER [10]. While no glycosylase mutant mammalian cell lines are available, *Escherichia coli* mutants, which are tag$^-$ AlkA$^-$, exhibit hypersensitivity to NMs (O'Connor T, unpublished data). Furthermore, we have demonstrated an increased expression of ANPG in malignant B lymphocytes from patients with NM resistant chronic lymphocytic leukemia (CLL) as compared with those from sensitive patients [3].

More direct evidence of the involvement of ANPG in CNU resistance comes from studies which have shown that the human ANPG can induce resistance to CNUs when overexpressed in *E coli* ([11], O'Connor T, unpublished data). CNU toxicity is believed to result from interstrand cross-links that form between the N^1 position of guanine and the N^3 position of cytosine [12]. However, these cross-links represent only 3% of total alkylation following

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§ Abbreviations: ANPG, alkyl-*N*-purine DNA glycosylase; BCNU, *bis*-chloroethylnitrosourea; BER, base excision repair; CHO, Chinese hamster ovary; CCNU, cyclohexyl chloroethylnitrosourea; CNU, chloroethylnitrosourea; MGMT, O^6-methylguanine DNA methyltransferase; MLN, melphalan; MMS, methyl methanesulfonate; NER, nucleotide excision repair; and NM, nitrogen mustard.

treatment with CCNU, while up to 16% of the lesions occur at the N^7 position of guanine. Such N^7 lesions are believed to be toxic, and these altered bases can be released by purified AlkA (the *E. coli* counterpart to ANPG) [13]. Enhanced ANPG activity was associated with CNU resistance in a glioma cell line [2]. This increased activity correlated with decreased accumulation of N^7-guanine-CNU lesions, indicating that these lesions may be a determinant in toxicity [14].

As ANPG is responsible for the initial step in BER, it is likely that this enzyme represents the rate-limiting step in repair of NM and CNU lesions. It was therefore of interest to determine what effect overexpression of this enzyme would have on cellular sensitivity to the bifunctional alkylating agents BCNU and MLN. To this end, we have transfected wild-type CHO-AA8 cells with an expression vector encoding the human ANPG. We have also transfected the NER deficient CHO line, UV20, with the same vector in order to assess the role of the glycosylase in the absence of NER.

MATERIALS AND METHODS

Drugs and cell culture. Stock MLN (Sigma) was dissolved in 75% ethanol, 0.032 N HCl at a concentration of 6.55 mM. BCNU was dissolved in 10 mM sodium citrate (pH 4) at a concentration of 15 mM. Immediately prior to addition of drug to the culture medium, the drug stocks were diluted into Dulbecco's phosphate-buffered saline. CHO cell lines (AA8 and UV20) were maintained in α-Minimum Essential Medium, 10% fetal bovine serum, 10 μg/mL gentamycin. Transfected cell lines were maintained in MAXTA as described by Weber *et al.* [15] (α-MEM with 10% fetal bovine serum, 100 μg streptomycin, 100 U penicillin, 10 μg mycophenolic acid, 25 μg adenine, 250 μg xanthine, 10 μg thymidine, 0.25 μg amethopterin, and 440 μg glutaminel/mL). All cell culture reagents were obtained from Gibco (Grand Island, NY), and tissue culture plasticware was obtained from NUNC (Gibco). Cells were found free of mycoplasma at the end of the experimentation period by staining with Hoechst 33258 and visualization under ultraviolet light.

Cytotoxicity assays. Cytotoxicity assays were performed as described [4]. Aliquots of 150 cells were seeded into 60 mm petri dishes and given 4 hr to attach to the substratum prior to treatment. The cells were then treated with either MLN or BCNU and incubated for 6 days. Colonies were fixed and stained in 10% trichloroacetic acid, 0.2% sulforhodamine B (Sigma) and washed with 1% acetic acid. Routinely the colony-forming efficiency of the UV20, AA8, and transfected cells was 70–80%.

Transfection experiments. Plasmids were prepared by alkaline lysis and purified on cesium chloride gradients [16]. The full length cDNA encoding the human ANPG [17] was subcloned into the pSVK3 expression vector (Pharmacia) yielding pSVANPG. Cells were transfected with 18 μg of either pSVANPG or pSVK3 and 2 μg of pSV2gpt using the calcium phosphate technique as described [4]. Stock cells were maintained under a constant selection of MAXTA, and cells used in cytotoxicity assays were cultured in non-selective medium for 3 days as described [4].

Glycosylase assays. Approximately 10^7 cells were lysed in 150 μL of extraction buffer [50 mM Tris–HCl, pH 7.6, 1 mM EDTA, 10 mM dithiothreitol (DTT), and 0.2% Triton X-100] on ice. The lysates were centrifuged at 12,000 *g* at 4° for 10 min in a microfuge. Supernatants were stored at −70°. Nuclear fractions were prepared as described [18]. Cells were washed twice with ice-cold PBS and resuspended in hypotonic buffer (20 mM Tris–HCl, pH 7.1, 5 mM KCl, 1 mM $MgCl_2$ and 0.2 mM phenylmethylsulfonyl fluoride). The cells were allowed to swell on ice for 10 min and then were disrupted with 20 strokes using a pestle in a tight-fitting Dounce homogenizer. The cell suspension was centrifuged at 375 *g* for 10 min. The pellet was resuspended in hypotonic buffer and centrifuged again. The final pellet was then lysed in extraction buffer, and the supernatant was used to assay for glycosylase activity.

For the glycosylase assay, *Micrococcus lysodeikticus* DNA (Sigma) was alkylated with [^{3}H]-dimethyl sulfate (5 mCi; 1.6 Ci/mmol; New England Nuclear) and used as a substrate for the glycosylase enzyme. The reaction was carried out in 100 μL of reaction buffer (70 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM DTT, and 10^4 dpm of [^{3}H]methylated DNA) plus 0–20 μL of cellular extract and incubated at 37° for 60 min. The reaction was stopped by the addition of 10 μL of 2 M NaCl, 10 μL salmon sperm DNA (2 mg/mL) and 300 μL ethanol. The DNA was precipitated at −20° for 30 min and pelleted by centrifugation at 12,000 *g* for 10 min at 4°. Then, 300 μL of supernatant was removed for scintillation counting. The protein concentration for each extract was determined using the Bio-Rad protein assay (Bio-Rad Laboratories). The glycosylase assay was done using extracts from the same cells as in the cytotoxicity assay, to ensure that the glycosylase activity was not affected by culturing in non-selective medium.

RESULTS

While ANPG expression has been implicated in bifunctional alkylating agent resistance, this association has never been demonstrated clearly in mammalian cells. To do so, wild-type CHO AA8 cells were transfected with an expression vector containing the full length human ANPG cDNA. Previous results revealed that the human gene product is functional in CHO cells and recognizes both N^3-methyladenine and N^7-methylguanine lesions [8, 19]. Nuclear extracts were prepared from the transfected cells as evidence of nuclear localization. Activity profiles of nuclear preparations from the three clones used in this paper (AGLY-1, -2, and -3) are shown in Fig. 1. Also shown is the activity profile of a control cell line, which was transfected with the pSVK3 expression vector without the ANPG cDNA (A-CON). We screened our clones for resistance to the bifunctional alkylating agents MLN and BCNU. No increase in resistance was

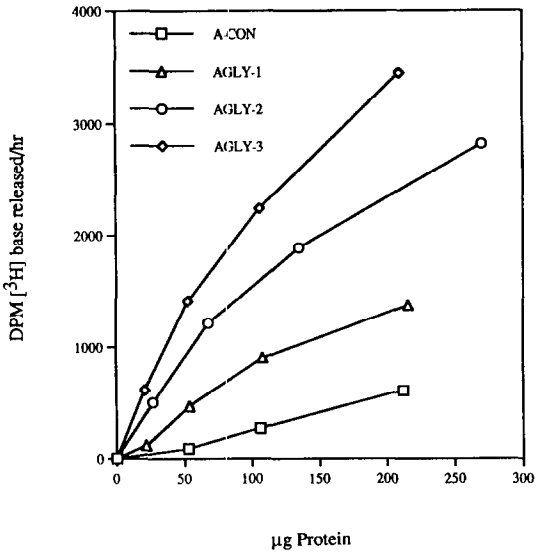

Fig. 1. Glycosylase activity in nuclear preparations from AA8 transfectants. AGLY-1, -2, and -3 = ANPG transfected cells; A-CON = pSVK3 transfected cells. The results are representative of a single experiment performed in duplicate. The activity was estimated from the initial slope of the curve and found to be 2.9 ± 0.35, 8.8 ± 2.3, 19.0 ± 3.7, and 41.3 ± 9.6 dpm of [^{3}H]-methylated bases released/hr/μg protein for A-CON, AGLY-1, -2, and -3, respectively (mean ± SEM; 3 independent experiments).

detected in any of the lines tested (Fig. 2). There were no differences between the control transfected cells and the parental AA8 cells in sensitivity to either MLN or BCNU (data not shown).

Both base excision and nucleotide excision repair have been implicated in resistance to alkylating agents. It is possible that in wild-type CHO cells, nucleotide excision repair dominates over the base excision pathway and, thus, changes in the base excision pathway are undetectable. Therefore we transfected the mutant UV20 cell line with the ANPG expression construct. UV20 cells lack a functional ERCC-1 gene and have an inhibited NER pathway. While these cells are 20- and 15-fold more sensitive to MLN and BCNU, respectively, as compared with AA8 cells, they are only 1.2-fold more sensitive to MMS, indicating a functional BER system. UV20 cells were transfected with the ANPG expression construct, and several lines were clonally derived. Figure 3 shows the activity profiles of nuclear extracts prepared from the clones used in this paper (UVGLY-1, -2, and -3) as well as a line transfected with pSVK3 (UV-CON). These cell lines displayed no differences in resistance to either MLN or BCNU (Fig. 4). The cell line UVGLY-2 seemed slightly more resistant to the effect of BCNU. However, the cell line UVGLY-3, which expresses higher glycosylase activity, seemed to be more sensitive. The inverse was true for MLN sensitivity. Thus, these small differences in alkylating agent sensitivity can be attributed to clonal heterogeneity and not to glycosylase activity.

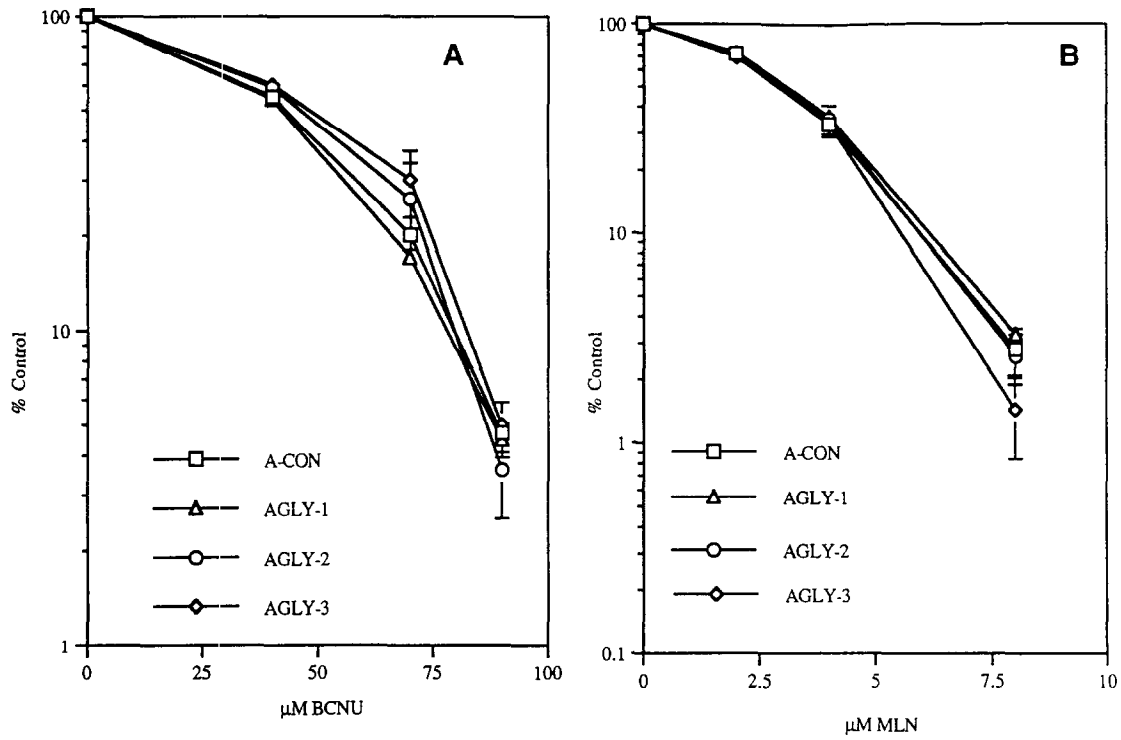

Fig. 2. Survival of AA8 transfectants following treatment with *bis*-chloroethylnitrosourea (A) and melphalan (B). AGLY-1, -2, and -3 = ANPG transfected cells; A-CON = pSVK3 transfected cells. Values are means ± SEM of 4–6 separate experiments performed in triplicate.

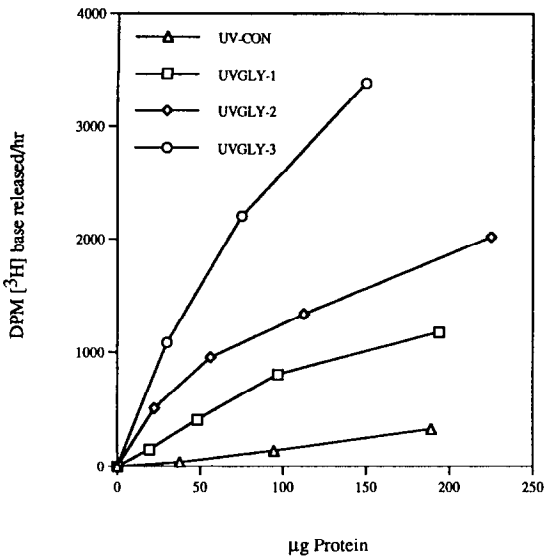

Fig. 3. Glycosylase activity in nuclear preparations from UV20 transfectants. UVGLY-1, -2, and -3 = ANPG transfected cells; UV-CON = pSVK3 transfected cells. The results are representative of a single experiment performed in duplicate. The activity was estimated from the initial slope of the curve and found to be 2.9 ± 0.35, 11.1 ± 1.4, 21.0 ± 2.0, and 40.0 ± 2.3 dpm of [^{3}H]-methylated bases released/hr/µg protein for UV-CON, UVGLY-1, -2, and -3, respectively (mean ± SEM; 3 independent experiments).

DISCUSSION

While enhanced DNA repair is responsible for resistance in certain tumors, the molecular mechanisms underlying this process are still undefined. The hypersensitivity of certain NER deficient cell lines to alkylating agents like BCNU and MLN has implicated NER in the process [1]. No glycosylase deficient mammalian cells exist; therefore, such an analogy is impossible. ANPG expression in glycosylase deficient bacteria corrects the hypersensitivity to mechlorethamine/BCNU, and ANPG expression in repair proficient *E. coli* confers resistance to BCNU, implying a role for BER in repair of these lesions in prokaryotes ([11]; O'Connor T, unpublished data). It has been shown that purified AlkA (the *E. coli* homolog of ANPG) can release bases from DNA substrates treated with CNUs [14]. We also have evidence that AlkA can release bases modified by NMs (O'Connor T, unpublished data). More importantly, human ANPG has been shown to release CNU specific lesions from DNA as well [11]. With respect to drug resistance, ANPG activity is increased in CLL lymphocytes resistant to NMs and in glioma cell lines resistant to CNUs [2, 3]. Recognition and release of the damaged base may be the key step in repair; therefore, we examined the effect of ANPG overexpression on sensitivity to bifunctional alkylating agents in CHO cells.

We transfected the NER proficient cell line, CHO-AA8, with an expression construct encoding the full length ANPG cDNA. The CHO cell lines were chosen for two reasons: (a) these cells are Mer$^-$, so

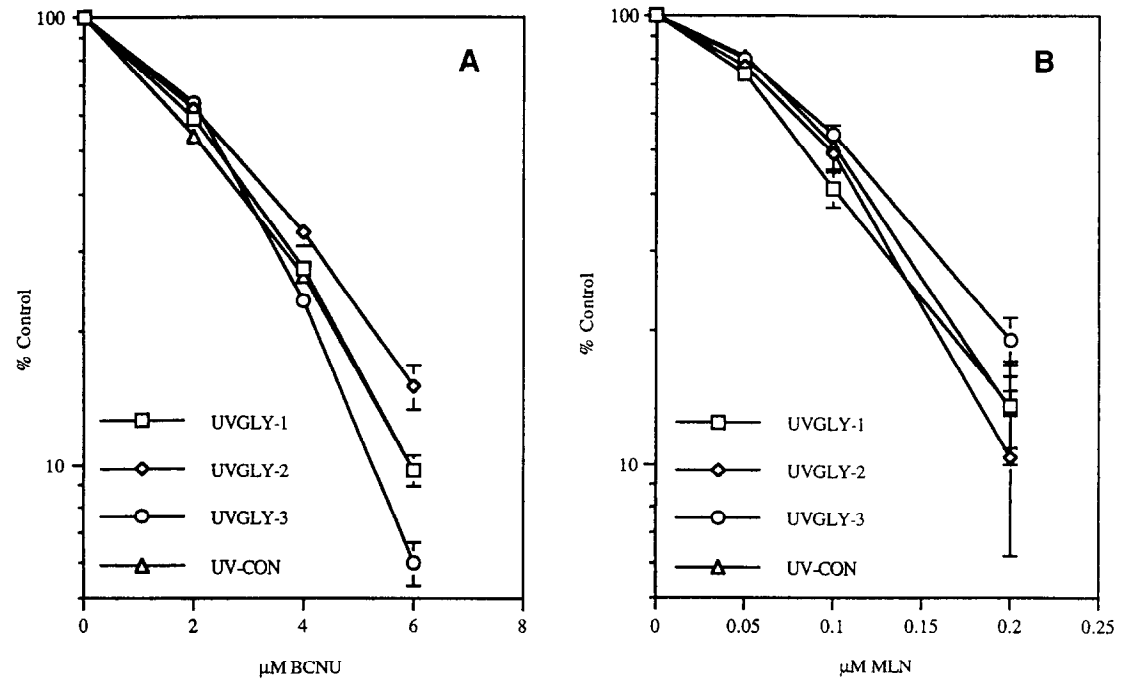

Fig. 4. Survival of UV20 transfectants following treatment with *bis*-chloroethylnitrosourea (A) and melphalan (B). UVGLY-1, -2, and -3 = ANPG transfected cells; UV-CON = pSVK3 transfected cells. Values are means ± SEM of 3–6 separate experiments performed in triplicate.

the results with BCNU will not be affected by MGMT [20], and (b) the availability of mutant lines allowed us to assess the role of the glycosylase in the absence of NER [1]. We developed clonally selected cell lines that expressed between 3 and 10 times the normal ANPG activity. None of these cell lines displayed increased resistance to BCNU or MLN. We believed that the NER pathway may predominate over BER in these cells, and therefore we chose an NER deficient cell line (CHO-UV20) in order to observe the effect of ANPG overexpression in the absence of NER. Again, the glycosylase transfected cells displayed no resistance to BCNU or MLN. It is possible that the Mer^- cells, which develop toxic N^1-guanine-N^3-cytosine cross-links, are much less sensitive to N^7-guanine lesions of BCNU than Mer^+ cells and, thus, increased repair of the latter lesions will not alter BCNU cytotoxicity in Mer^- cells.

In spite of these results, there remains strong evidence that BER may play a role in the repair of bifunctional alkylating agent damage. The *E. coli* data argue strongly that BER can be a determinant in resistance in prokaryotes. A CHO mutant line that displays hypersensitivity to NM and MLN is also hypersensitive to MMS [10]. As MMS lesions are repaired by BER, hypersensitivity to this agent indicates impairment in this repair pathway. This suggests that BER is required, at least in part, for wild-type resistance to MLN. The N^1-guanine-N^3-cytosine cross-link, which results from CNU alkylation, is believed to be responsible for the toxicity of these agents [12]. This cross-link is formed via an initial alkylation at the neighboring O^6 position of guanine. Expression of MGMT, which repairs lesions at O^6-guanine, in CHO cells can render them resistant to CNU [21]. However, the existence of other lethal lesions has been demonstrated [22]. $RecA^-$ *E. coli*, which exhibit hypersensitivity to both BCNU and ENU, can be adapted to nitrosourea exposure. The adapted cells are resistant to ENU, indicating proper repair of the O^6 lesion; however, they are still hypersensitive to the effects of BCNU [23]. O^6 Alkylation repair will prevent formation of the N^1-N^3 cross-link; thus, toxic lesions other than cross-links must also result from CNU alkylation. These lesions are likely to be N^7-guanine lesions, which represent the bulk of base alkylations following CNU treatment. N^7-CNU lesions have also been shown to be substrates for the AlkA enzyme and human ANPG [11, 13].

ANPG activity has been shown to be increased in both NM resistant CLL lymphocytes and CNU resistant gliomas [2, 3]. HPLC analysis of the cellular DNA from the glioma cells following BCNU treatment shows that both the N^7-lesions and N^1-N^3 cross-links are reduced, consistent with the observation of increased ANPG and MGMT activity [2, 14]. This suggests that, at least in gliomas, ANPG and MGMT may be members of a similar repair system that responds to alkylation damage. Evidence of this comes from studies that show that MGMT and ANPG transcripts are both inducible upon challenge with mitomycin C or *cis*-diamminedichloroplatinum(II), two DNA cross-linking agents [24, 25]. This evidence suggests that both ANPG and MGMT may act in a common pathway to render cells resistant to CNUs and NMs.

Expression of the human ANPG in tag^- $alkA^-$ *E. coli* strains can render these cells resistant to BCNU [11]. Furthermore, when $uvrA^-$, tag^- $AlkA^-$ *E. coli* (deficient in both NER and BER) are used, ANPG expression results in full complementation of BCNU sensitivity and partial complementation to mechlorethamine (O'Connor T, unpublished results). Thus, it appears that *E. coli*, which lack functional cross-link repair, can benefit from overexpression of ANPG. The lack of a similar effect in the hypersensitive UV20 cell line indicates that there is an important difference in the regulation of BER in mammalian cells. In spite of a 10-fold increase in ANPG activity in the UVGLY-3 cells, there was no increase in resistance to BCNU. One explanation for this is that a second protein may be required in eukaryotic cells which is not required for enhanced repair in prokaryotes. Another possibility is that the chromatin structure of mammalian cell DNA may limit the function of ANPG. Ibeanu *et al.* [19] suggested that in BER, repair of the abasic lesions may be limiting. In a recent report, overexpression of AP endonuclease in rat glioma cells did not produce resistance to MMS. Interestingly, reduction of AP endonuclease activity using antisense vectors rendered cells more sensitive to MMS, indicating that this protein can be limiting [26]. Greater understanding of the base excision repair process in mammalian cells will provide insight into this process. Unfortunately, ANPG deficient cell lines have not yet been identified, and therefore the involvement of ANPG in alkylating agent resistance is dependent upon the success of transfection experiments like the ones performed in this paper.

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