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Reduction of the toxicity and mutagenicity of aziridine in mammalian cells harboring the *Escherichia coli fpg* gene

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ABSTRACT

Aziridine (ethyleneimine) reacts with DNA *in vitro,* mainly at the N7 position of guanine and N3 of adenine, then imidazole ring opening of the modified guanine results in formation of formamidopyrimidine (FaPy) residues. The *Escherichia coli fpg* gene encodes a DNA glycosylase that removes FaPy residues from DNA. To determine whether aziridine produces FaPy lesions in mammalian cells we have expressed the *E.coli fpg* gene in CHO cells. The transfected cells, expressing high levels of the bacterial protein, are more resistant to the toxic and mutagenic effects of aziridine than the control population. Less DNA damage was measured by quantitative PCR analysis in transfected than in control cells treated with equimolar concentrations of aziridine. The results suggest that aziridine produces *in vivo* FaPy residues that could account for the deleterious effects of this compound.

INTRODUCTION

Chemical compounds induce a wide variety of lesions in DNA that can interfere with replication and transcription and, therefore, result in mutagenesis or cell death. Aziridine (ethyleneimine) is an alkylating agent produced in large amounts in industry (1). This compound has been shown to be toxic and mutagenic in various biological systems and to produce chromosome abberations and sister chromatid exchanges in human cells (reviewed in 2). However, there are few data in the literature concerning the nature of the DNA damage produced by this compound *in vivo* and, hence, concerning the mechanisms implicated in repair of these lesions.

It has been shown that aziridine reacts *in vitro* with guanine and guanosine to form an *N*7-aminoethyl derivative (3). This product exhibits an unusual tendency to undergo imidazole ring opening (4) and the reaction of guanosine with aziridine results in the formation of two main products: imidazole ring-opened *N*7-alkylguanosine and *N*1-alkylguanosine, accounting for 80 and 14% of all adducts respectively (4). Protonated aziridine also reacts with the N3 nitrogen of adenine, although this site is predicted to be less reactive than N7 of guanine (5). In *Escherichia coli* ring-opened guanine and ring-opened adenine, formed by alkylating agents , are repaired by a specific formamidopyrimidine-DNA glycosylase, the Fpg protein (6). This protein is encoded by the *fpg* (or *MutM*) gene (7), which, besides its glycosylase activity, also possesses AP-nicking (6) and dRPase activities (8).

In order to check whether the aziridine-induced DNA damage formed in mammalian cells could be recognized and repaired by formamidopyrimidine-DNA glycosylase activity we have overexpressed the bacterial Fpg protein in Chinese hamster ovary (CHO) cells. The lethal and mutagenic effects of aziridine were measured in CHO cells expressing or not the Fpg protein. The number of lesions formed in a specific genomic region was measured by quantitative PCR (9,10) in control and transfected cells. The results show that overexpression of the bacterial Fpg protein protects the cells against the deleterious effects of aziridine and decreases the lesion frequency produced by this coupound.

MATERIAL AND METHODS

Cell culture

CHO cells were grown in Dulbecco's medium supplemented with 5% fetal calf serum and 5% horse serum. They were transfected by electroporation, using a BioRad gene pulser apparatus as described (11). The transfected cells were grown in G418-containing medium (750 μg/ml) until appearance of clones.

The survival of aziridine-treated cells was measured by incubating exponentially growing cells for 30 min at 37°C in culture medium supplemented with 2% serum and increasing concentrations of the drug. They were then rinsed, trypsinized and aliquots of the suspension were cultured in normal medium until appearance of clones.

For mutagenicity determination the cells were incubated with aziridine as described above, then grown in fresh medium for 8–11 days to allow expression of the mutant phenotype. They were then subcultured in normal medium for survival determination or in the presence of 6-thioguanine (2.5 μg/ml) to measure the mutation frequency.

To measure DNA synthesis the cells were incubated with various aziridine concentrations for 30 min, rinsed and then grown for 2 h in normal medium. They were then grown for 30 min in the presence of [^{3}H]thymidine and the specific activity of the cellular DNA determined as already described (12).

Plasmid construction

The coding sequence of the *E.coli fpg* gene (809 bp) was excised with *Hin*dIII and *Pvu*II from plasmid fpg220 (6). After purification by gel electrophoresis the gene was ligated in the *Hin*dIII site of the

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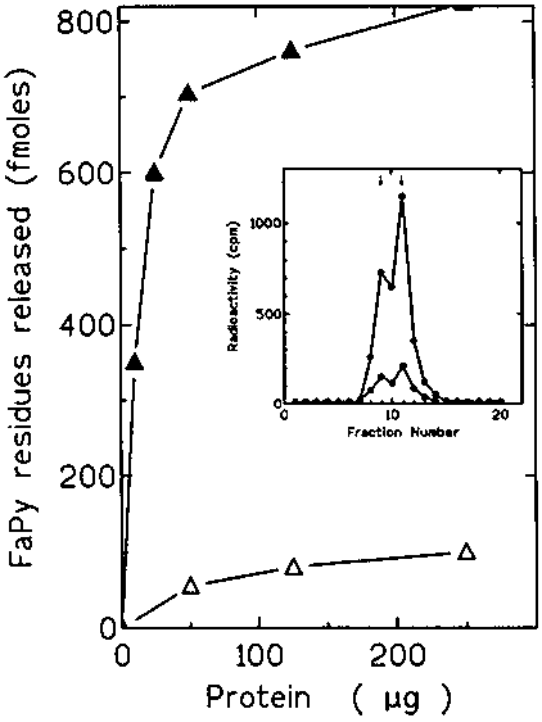

Figure 1. FaPy-DNA glycosylase activity in cells transfected with plasmid psV2-FPG. Cell extracts were incubated with [^{3}H]FaPy–poly(dG·dC) for 30 min at 37°C. For details see Materials and Methods. Δ, control cells; ▲, transfected cells. (Inset) Analysis by HPLC of the reaction products from control (○) and transfected cells (●). The arrows indicate the position of the two FaPy rotamers.

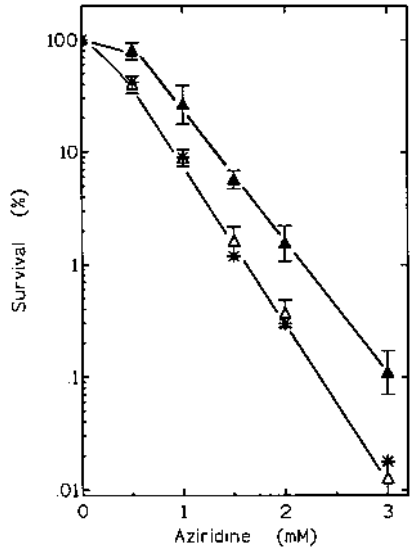

Figure 2. Survival curves for aziridine-treated cells. Cells were incubated for 30 min with increasing aziridine concentrations, then plated for survival. Δ, Control cells; ▲, cells transfected with the *fpg* gene; ✷, cells transfected with *APDG* cDNA. Results are the mean ± SD of three separate experiments.

psV2-neo vector. The plasmid carrying the insert was designated psV2-fpg. A psV2-neo vector carrying *APDG* cDNA (encoding rat *N*3-methyladenine-DNA glycosylase) (13) was constructed and designated psV2-APDG.

Determination of formamidopyrimidine-DNA glycosylase activity

Cells were suspended (10^8 cells/ml) in a buffer containing 70 mM HEPES, 100 mM KCl, 2 mM EDTA, 1 mM DTT and 10% glycerol. They were disrupted by sonication at 0°C in the presence of proteases inhibitors (leupeptin, aprotinin and antipain, 2µg/ml each). Cell debris was removed by centrifugation (10 000 *g*, 5 min, 4°C). Increasing amounts of cell extracts were incubated (final volume 100 µl) for 30 min at 37°C with [^{3}H]formamidopyrimidine (FaPy)–poly(dG·dC) prepared as previously described (6). The radioactivity present in the ethanol-soluble fraction was quantitated by scintillation spectroscopy. Characterization of the two rotameric forms of FaPy residues (14) in the ethanol fraction was by HPLC analysis after addition of authentic markers, using a C18-µBondapack column eluted with 50 mM ammonium phosphate containing 5% methanol.

Quantitative PCR analysis

Cells were lysed in 0.5 M NaCl, 0.05 M EDTA, 0.05 M Tris, pH 7.5, by addition of SDS (2% final concentration). The lysate was treated for 2 h at 55°C with proteinase K (200 µg/ml; Boehringer, Mannheim), then the DNA purified as described (9). Exon 9 (723 bp) or exons 2–3 (3 kb) of the *HPRT* gene were amplified using the primers described by Rossiter *et al.* (15) and a DNA Thermal Cycler (Perkin Elmer Cetus). Amplification products were radioactively labeled by incorporation of [^{32}P]dCTP (16) during the exponential increase in the PCR products (between 26 and 30 cycles) and were analyzed by electrophoresis in 1.8% agarose gels. Quantitation of the PCR products was performed by TCA precipitation and scintillation counting as described (17). The number of lesions per strand was calculated as $-\ln(A_d/A_o)$, where A_d is c.p.m. incorporated in damaged DNA and A_o is c.p.m. incorporated in non-damaged DNA template (10).

RESULTS

FaPy-DNA glycosylase activity in transfected cells

Among the different G 418-resistant clones obtained after transfection of CHO cells with plasmid psV2-fpg some expressed a high level of FaPy-DNA glycosylase activity, which was ~40 times higher than the endogenous level for CHO cells (Fig. 1). Analysis of the reaction products by HPLC showed removal of FaPy residues (Fig. 1, insert). Inhibition of the activity in cell extracts incubated with specific antibodies raised against the bacterial Fpg protein confirmed that the increased activity was due to expression of the bacterial gene (18).

Effect of *fpg* gene expression on aziridine toxicity and mutagenicity

The survival of CHO cells was measured after exposure to increasing aziridine concentrations. The survival curves showed that the transfected cells (Fig. 2) were more resistant than the controls to the lethal effect of this compound. It should be noted that the growth rate was identical in the two cell populations and that no significant modification of cell survival was observed in cells transfected with the control psV2-neo vector (Table 1). To check that the enhanced resistance was specifically related to expression of the Fpg protein CHO cells were transfected with psV2 vector carrying *APDG* cDNA. This cDNA was used because it expresses rat *N*3-methyl-

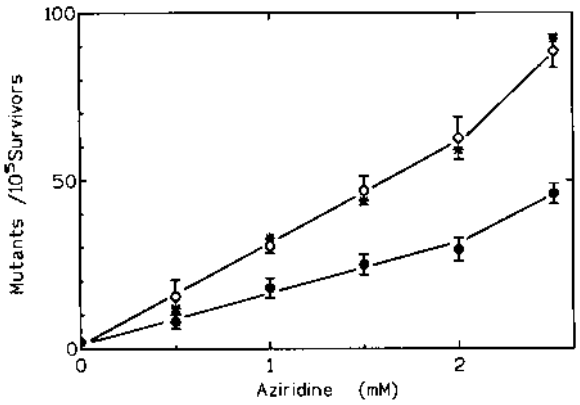

Figure 3. Mutation frequency in aziridine-treated cells. The cells were treated with aziridine for 30 min, then grown for 10 days in normal medium before selection for 6-thioguanine resistance. ○, Control cells; ●, cells transfected with the *fpg* gene; ✶, cells transfected with *ADPG* cDNA. Results are the mean value ± SD of three separate experiments.

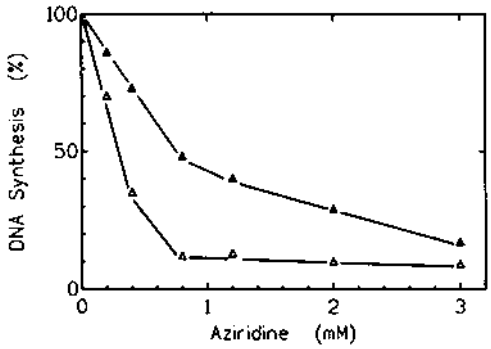

Figure 4. DNA synthesis in aziridine-treated cells. CHO cells were incubated for 30 min in the presence of aziridine, rinsed and grown for 2 h in normal medium. They were then incubated for 30 min in the presence of [^{3}H]thymidine. For details see Materials and Methods. △, Control cells; ▲, transfected cells.

adenine-DNA glycosylase, which specifically removes *N*3-alkyladenine and *N*7-alkylguanine residues from DNA (12): the transfected cells had an APDG activity ~10 times higher than the control value (Table 1), but this enhanced activity did not modify cell sensitivity to the lethal effect of aziridine (Fig. 2 and Table 1). It should be recalled that expression of *APDG* cDNA could increase cell resistance to the toxic effect of MMS or EMS (11).

Results presented in Figure 3 show that aziridine is a potent mutagenic compound in mammalian cells. To check whether the mutagenic lesion(s) produced by this compound were repaired by Fpg protein the number of mutations in the *HPRT* locus was measured in control and transfected cells. The results showed that the aziridine-induced mutation frequency was reduced in *fpg* gene-expressing cells (Fig. 3). However, when control experiments were performed with cells transfected with the psV2-APDG vector no decrease in aziridine-induced mutation frequency was observed (Fig. 3).

Frequency of aziridine-induced DNA damage

It has been shown that the lethal effects of alkylating agents are due to the formation of DNA base modifications which block DNA replication, e.g. *N*3-methyladenine residues (19). Therefore, experiments were designed to check whether the lethal effect of aziridine was related to formation of DNA blocking lesions and whether these lesions were recognized by Fpg protein. When DNA synthesis was measured in CHO cells treated with different aziridine concentrations a dose-dependent decrease was observed and synthesis was almost totally abolished in control cells treated with 1 mM aziridine. However, higher aziridine concentrations were required to decrease DNA synthesis in *fpg*-expressing cells (Fig. 4), suggesting that the expressed protein removes an aziridine-induced blocking lesion. This arrest of DNA replication by the drug-induced lesions was confirmed by PCR amplification of genomic DNA. The same amounts of DNA (500 ng), isolated from control or transfected cells exposed to increasing aziridine concentrations, were amplified. Amplification of the *HPRT* gene (exon 9) decreased in aziridine-treated cells in a dose-dependent manner, becoming undetectable under our experimental conditions (Fig. 5a). It has been shown that measurement of incorporated radioactivity in amplified DNA allows determination of the number of lesions in a DNA fragment under experimental conditions where amplification is proportional to the template DNA concentration and to the number of cycles (9,10). We used such conditions to calculate the lesion frequency (Fig. 5b): it increased linearly with the aziridine dose, but was lower in the case of transfected compared with control cells. Based on the slope of the best fit line, the lesion frequencies were 0.68 and 0.21 lesions/723 bp oligonucleotide/2 mM aziridine in control and transfected cells respectively. Similar results were obtained using the 3 kb fragment, as in this fragment 2.75 and 0.72 lesions were calculated in control and *fpg*-expressing cells treated with 2 mM aziridine respectively.

Table 1. FaPy-DNA and APDG-DNA glycosylase activities and aziridine sensitivity in control and transfected cells

Cells	FaPy-DNA glycosylase activity[a]	APDG-DNA glycosylase activity[b]	D_{10} dose (mM)[c]
CHO (control)	0.21	0.50	0.90
CHO + pSV2-neo	0.22	0.48	0.92
CHO + pSV2-APDG	0.21	5.25	0.85
CHO + pSV2-FPG	7.88	0.51	1.30

[a]pmol FaPy residues removed/mg protein/30 min at 37°C.
[b]pmol *N*3-methyladenine residues removed/mg protein/30 min at 37°C.
[c]Aziridine concentration reducing the cell survival to 10% of the control value.

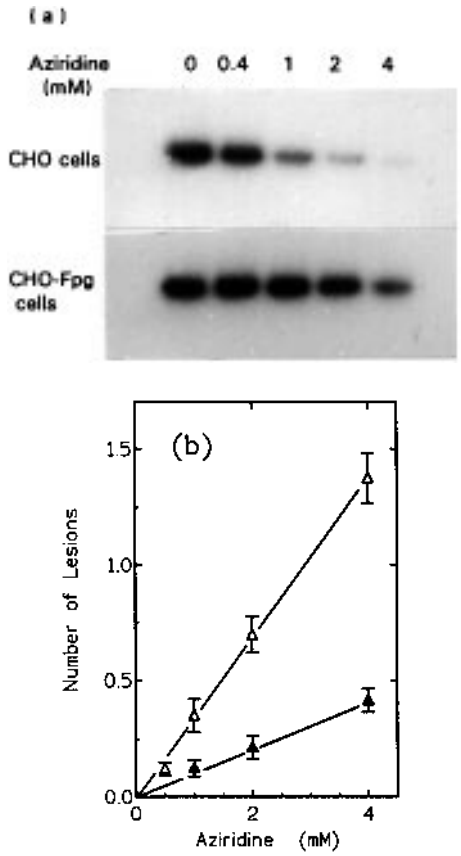

Figure 5. PCR amplification of the *HPRT* gene from aziridine-treated cells. Cells were incubated for 1 h with increasing aziridine concentrations, then the DNA was immediately isolated. PCR reactions were performed as described in Materials and Methods. (**a**) The PCR products corresponding to the *HPRT* gene (exon 9) from CHO or CHO-FPG cells were analyzed in 1.8% agarose gels. (**b**) The radioactivity associated with the amplified DNA was determined and used to calculate the number of lesions in the DNA fragment. Results are the mean value of two separate experiments. Δ, Control cells; ▲, CHO-FPG cells.

DISCUSSION

As aziridine has been shown to be a toxic and mutagenic compound (reviewed in 2), it was of interest to look for the mechanism implicated in repair of aziridine-induced damage in mammalian cells. This compound produces mainly ring-opened *N*7-guanine (4) and reacts with the N3 of adenine in DNA *in vitro* (5). These ring-opened bases are repaired in *E.coli* by Fpg protein (6). Since the mammalian counterpart of the *fpg* gene has not yet been cloned, it is difficult to assess the role of aziridine-induced DNA damage in mammalian cells. The mechanisms by which a number of diverse types of DNA damage are repaired are closely similar in *E.coli* and mammalian cells. This implies that the biological consequences of a particular unrepaired lesion, mutation or cell death, will be similar in both cell types. Therefore, we have studied repair of aziridine-induced damage in CHO cells by expressing the *E.coli fpg* gene.

Expression of the bacterial Fpg protein increases the resistance of CHO cells to the toxic and mutagenic effects of aziridine, suggesting that this protein removes aziridine-induced damage from cellular DNA. However, expression of this protein does not change cell sensitivity to MMS (Laval, unpublished results). This suggests that the biological effect of Fpg protein in the cells is due neither to its AP-nicking activity nor to its dRPase activity. Furthermore, the damage produced by aziridine is not recognized by rat *N*3-methyl-adenine-DNA glycosylase, as expression of this protein does not modify cell sensitivity to aziridine. As already described for HeLa cells (20), aziridine decreases DNA synthesis, suggesting formation of DNA lesions which are a block to DNA replication. The aziridine concentration which blocks DNA synthesis (1 mM) 2 h after treatment is not highly toxic to the cells. This suggests that even control cells can sustain a level of aziridine-induced DNA damage and this result is expected, because CHO cells possess a FaPy-DNA glycosylase activity. We have used the quantitative PCR technique to measure the number of replication blocking lesions in DNA of aziridine-treated cells, as it has been shown that PCR could be used to quantify damage induced in DNA by various agents, e.g. *cis*-platinum and UV light (9), even in a small gene segment (17). Using this analysis we could detect damage in aziridine-treated CHO cells and show that expression of the *E.coli fpg* gene decreased the number of lesions present in cellular DNA. However, it is possible that a low number of lesions are by-passed by *Taq* polymerase and therefore are not detected in our assay, lowering the number of lesions measured in the DNA of the treated cells. *In vitro* ring-opened *N*7-guanine residues on the template strand of a DNA molecule prevent movement of the replication fork and block DNA synthesis one base before the lesion (21). As Fapy residues are formed by aziridine (4) and repaired by the Fpg protein (6) they are good candidates to be one form of lethal damage produced by aziridine *in vivo*.

The presence of Fapy residues in M13 phage DNA correlates with a decrease in transfection efficiency and in mutagenicity when transfected into SOS-induced *E.coli* cells (22). In phage treated with dimethyl sulfate and alkali, treatment that produces FaPy residues, most mutations were found not at G, suggesting that FaPy–guanine is not mutagenic, but at A, yielding A→G transitions (22). These results suggest the formation of a mutagenic adenine derivative. As the mutagenic effect of aziridine decreases in CHO cells expressing Fpg protein, a modified adenine could be one form of damage responsible for the mutagenic properties of aziridine.

In conclusion, the Fpg protein removes two types of lesions from the DNA of aziridine-treated cells. (i) Lesions which are a block to DNA replication and are probably responsible for the lethal effect of this compound. Due to the specificity of the Fpg protein and the properties of FaPy–guanine residues, these residues are a good candidate for this lethal lesion. (ii) Lesions which are mutagenic and are also recognized by the Fpg protein. It has been suggested that FaPy–adenine residues could occur and be mutagenic in phage treated with alkylating agents and alkali (22). This damage is repaired by the Fpg protein and could, therefore, be responsible for the mutagenic effect of aziridine in mammalian cells.

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