[CANCER RESEARCH 56, 3721–3724, August 15, 1996]

Increased Resistance to *N,N′,N″*-Triethylenethiophosphoramide (Thiotepa) in Cells Expressing the *Escherichia coli* Formamidopyrimidine-DNA Glycosylase[1]

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ABSTRACT

Thiotepa (*N,N′,N″*-triethylenethiophosphoramide) is an alkylating agent used in cancer chemotherapy. A reaction pathway by which thiotepa alkylates purified DNA involves hydrolysis to aziridine, which forms N_7-aminoethyl guanine and aminoethyl adenine. These lesions are repaired in *Escherichia coli* by the formamidopyrimidine-DNA glycosylase (Fpg) protein. To determine whether such lesions are formed by thiotepa in mammalian cells, we have overexpressed the *E. coli* Fpg protein in Chinese hamster ovary cells. The transfected cells were more resistant to the lethal and mutagenic effects of thiotepa than the parental cells. The number of replication-blocking lesions formed by thiotepa, measured by quantitative PCR analysis, was lower in the transfected cells. These results show that expression of the Fpg protein increases the cell resistance to thiotepa and suggest that this compound produces ring-opened guanines, which are involved in its cytotoxic action.

INTRODUCTION

Alkylating agents are widely used in cancer therapy. Among them, thiotepa,[3] which contains a four-coordinated phosphorus atom and three aziridine moities, has been in use since the early 1950s (1). In recent years, there has been renewed interest in its use, in conjunction other alkylating agents, in the treatment of a variety of tumors (2).

However, the mechanism by which thiotepa alkylates the cellular DNA is not well defined, and little is known about the mechanisms involved in the repair of thiotepa-induced DNA damage in mammalian cells. Two different pathways have been proposed for the reactivity of this compound with DNA. Alkaline elution experiments suggest that thiotepa-induced cell death is due to the formation of cross-links within DNA (3), these cross-links being formed on direct nucleophilic ring opening of two aziridinyl groups. A second pathway involves hydrolysis of thiotepa to aziridine (ethylene imine), due to a nucleophilic attack of water at the phosphorus atom and cleavage of a N-P bond. Experiments have shown that thiotepa reacts with purified DNA, resulting in depurination and formation of aminoethyl adducts of guanine and adenine (4). It is known that N_7-aminoethyl guanosine readily decomposes by imidazole ring opening and depurination (5). Although the release of aziridine is dependent on the pH (4), the formation of aziridine from thiotepa has been suggested in cultured L1210 cells (6).

Ring-opened guanine residues are repaired in *Escherichia coli*, independently of the N_7 substituent, by the Fpg protein (7). This protein is encoded for by the *fpg* gene (8) and possesses three different activities; it acts as a glycosylase and has nicking activity at AP sites (9) and dRPase activity (10). Similar DNA glycosylase activity has been described in mammalian cells (11), but the cDNA coding for this activity has not yet been cloned.

To determine whether ring-opened guanine residues were formed in the DNA of cells treated with thiotepa, we have expressed the *E. coli fpg* gene in CHO cells and measured the influence of the Fpg protein on the cell sensitivity to thiotepa. In this article, we show that cells expressing the bacterial protein are more resistant to the toxic effect of this compound and are able to remove the replication-blocking lesions formed by the drug, as determined by QPCR analysis, more efficiently than control cells.

MATERIALS AND METHODS

Plasmid Construction. The fpg 220 plasmid, which encodes the *E. coli fpg* (8), was cut by *Hin*dIII and *Pvu*II to obtain the *fpg* coding sequence (809 bp). The gene was purified by electrophoresis then ligated in the psV2neo vector, yielding the psV2-fpg plasmid. A similar construct was made with the APDG cDNA (encoding rat N_3-methyladenine-DNA-glycosylase; Ref. 12) and called psV2-APDG.

Cell Culture and Transfection. CHO cells were grown in DMEM supplemented with 5% FCS and 5% horse serum. They were transfected by electroporation, using a Bio-Rad gene pulser apparatus, as described (13). Clones resistant to G418 (750 μg/ml) were selected. Cells expressing the *E. coli fpg* gene were called CHO-fpg, and cells expressing the mammalian APDG cDNA were called CHO-APDG.

Concentrated solutions of thiotepa (Lederle Laboratories, the Netherlands; 5 mg/ml) were prepared in water and used immediately. Thiotepa cytotoxicity was measured by incubating exponentially growing cells in culture medium containing increasing amounts of the drug. After 1 h at 37°C, the cells were rinsed, trypsinized, and subcultured until clones developed.

Mutation frequency at the *hprt* locus was measured by incubating the cells (1×10^6 cells/75-cm^2 flask) for 1 h with increasing amounts of thiotepa. The cells were then rinsed and grown for 6 days in fresh medium to allow expression of the mutant phenotype. They were trypsinized and subcultured either in normal medium for counting viable cells or in DMEM supplemented with 10% dialyzed FCS and 6-thioguanine (2.5 μg/ml) to determine the number of mutants (10^5 cells were plated per dish, and six dishes were used per drug concentration).

Fpg Activity. This activity was determined as described previously (14). Briefly, cells were suspended in a buffer containing 70 mM HEPES, 100 mM KCl, 2 mM EDTA, 1 mM DTT, and 10% glycerol (10^8 cells/ml). Cell extracts were prepared by sonication at 0°C in the presence of protease inhibitors and centrifugation. Increasing amounts of cell extracts were incubated, in a final volume of 100 μl, with [^{3}H]FaPy-poly[d(G-C)], prepared as described previously (8), for 30 min at 37°C. After precipitation, the ethanol-soluble radioactive products were quantified, and the two FaPy rotamers were characterized by HPLC (15).

QPCR Analysis. The cells were incubated for 3 h with increasing amounts of thiotepa prior to cellular DNA purification. Cells were lyzed in 0.5 M NaCl, 0.05 M EDTA, and 0.05 M Tris (pH 7.5) by the addition of SDS (2% final concentration). The lysate was treated for 2 h at 55°C with proteinase K (200 μg/ml; Boehringer Mannheim), and then the DNA was purified as described (16). The gene target for PCR amplification was exon 9 of the *HPRT* gene. Primers were purchased from Pharmacia and were of the following sequences: 5′-CCT TGT TTG GTA GGA ACC AG-3′ from the coding strand and 5′GTA CTG TCT CAA GTA GAC AG-3′ from the noncoding strand, yielding an 884-bp PCR product.

Received 3/29/96; accepted 6/18/96.

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[1] This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale; ARC (Villejuif); the Special Trustees of Middlesex Hospital, London; and the Cancer Research Campaign (CRC), United Kingdom.

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[3] The abbreviations used are: Thiotepa, *N,N′,N″*-triethylenethiophosphoramide; Fpg, formamidopyrimidine-DNA glycosylase; CHO, Chinese hamster ovary; FaPy, formamidopyrimidine; HPLC, high-performance liquid chromatography; QPCR, quantitative PCR.

Amplifications were carried out in triplicate with 0.4 μg DNA from each treatment group. The total volume of the PCR mixture was 100 μl, containing 35 pmol of each primer, 2.5 units of Red Hot *Taq* polymerase (Advanced Biotechnologies), 2 μCi [γ-^{32}P]dCTP, 180 μmol of each dCTP, dGTP, dATP, and dTTP, 1.25 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl (pH 8.3). Cycles were performed in an M. J. Research, Inc. PTC-100 Hot Bonnet thermocycler, without oil overlay, as follows: denaturation at 94°C for 4 min, then 30 cycles of 94°C for 1 min, 55°C for 1 min, and 73°C for 1 min, with a final elongation cycle of 73°C for 4 min. Under these conditions, the PCR is still in an exponential phase when stopped.

To measure the amount of PCR product, 40-μl aliquots of each sample were precipitated with 1 ml 5% (w/v) trichloroacetic acid and 20 mM tetrasodium PP_i, and the precipitate was captured on Whatman GFC filter disks using a vacuum manifold (Millipore). The filters were washed with 5% trichloroacetic acid, then with ethanol, and the radioactivity was counted in a Beckman LS1800 scintillation counter (17).

RESULTS

Characterization of the Transfected Cells. Numerous G418-resistant clones were obtained after transfection of CHO cells with the psV2-fpg plasmid. These clones were grown separately, and the Fpg activity was measured in different clones. One of them, named CHO-fpg, expressed a high level of glycosylase activity; the release of FaPy residues by cell extracts was checked by HPLC analysis of the reaction products (Fig. 1), and the activity measured in the transfected cells was about 40-fold higher than the constitutive level for CHO cells (Table 1). As described previously (14), specific antibodies were used to check that this increased activity was actually due to the expression of the bacterial gene within the cells (data not shown). The expressed Fpg activity was measured repeatedly and was found to be stable in the transfected cells.

Sensitivity of Control and Transfected Cells to Thiotepa. Cell survival was measured after exposure to increasing concentrations of thiotepa. It should be noted that the duration of the cell cycle and the plating efficiency were identical in control and transfected cells. The survival curves show that the transfected cells were more resistant to the lethal effect of thiotepa than the control population (Fig. 2). When CHO cells were transfected with the psV2-APDG plasmid, which expresses the rat N_3-methyladenine-DNA-glycosylase, there was no modification of the cell survival (Table 1), although the 3-methyladenine-glycosylase activity was 10-fold higher in the transfected compared with control cells (data not shown). This protein specifically removes the N_3-alkyladenine and N_7-alkylguanine residues from alkylated DNA (12). Control experiments were also carried out with CHO cells transfected with the psV2neo plasmid; these cells had the same sensitivity to thiotepa as the parental CHO cell line (Table 1). This suggests that expression of the *E. coli* Fpg protein specifically reduces the cytotoxic effect of thiotepa in the transfected cells.

To assess the effects of fpg expression on mutation induction, mutation frequencies at the *hprt* locus were measured after exposure to thiotepa. The spontaneous mutation frequencies were similar in control and transfected cells. When the cells were exposed to the drug, the induced mutation frequency was slightly decreased in Fpg protein-expressing cells (Table 2), suggesting that this protein repairs a lesion that contributes to the overall mutation frequency induced by thiotepa.

Detection of Thiotepa-induced DNA Damage by QPCR. It is known that ring-opened guanine residues are a block to DNA replication *in vitro* (18). We have previously shown that adducts formed on DNA by alkylating agents can be detected quantitatively by PCR (17). The technique of QPCR was used to determine the number of polymerase-blocking lesions formed on an 884-bp DNA fragment of the *HPRT* gene, following thiotepa treatment of parental and fpg-expressing cells.

Table 1 *Sensitivity to thiotepa and Fpg activity in control and transfected CHO cells*
Results are the mean values ± SD of three separate experiments.

Cells	Fpg activity[a]	LD_{10}[b] (mM)
CHO (control)	0.21 ± 0.04	0.55 ± 0.05
CHO-fpg	7.88 ± 0.50	1.46 ± 0.08
CHO-APDG	0.21 ± 0.05	0.56 ± 0.04
CHO + psV2neo	0.22 ± 0.04	0.55 ± 0.07

[a] Picomoles of FaPy residues released by 1 mg protein in 30 min at 37°C.
[b] Thiotepa concentrations reducing the survival to 10% of the control value, as determined by regression analysis from data of the survival curves.

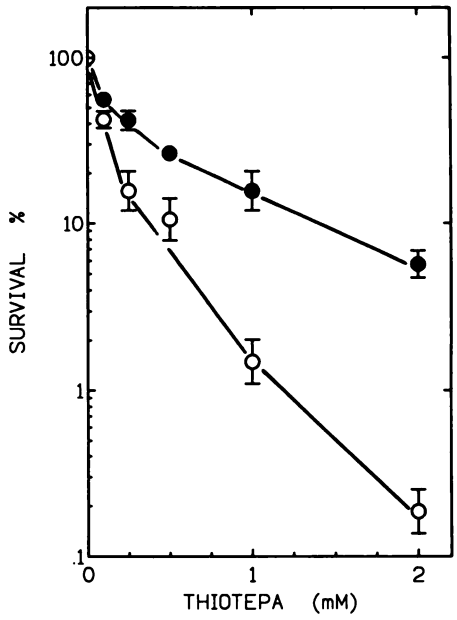

Fig. 2. Survival curves for CHO cells exposed to thiotepa. Control (○) or CHO-fpg (●) cells were incubated for 1 h with increasing thiotepa concentrations. Results are the mean values of three separate experiments. *Bars*, SD.

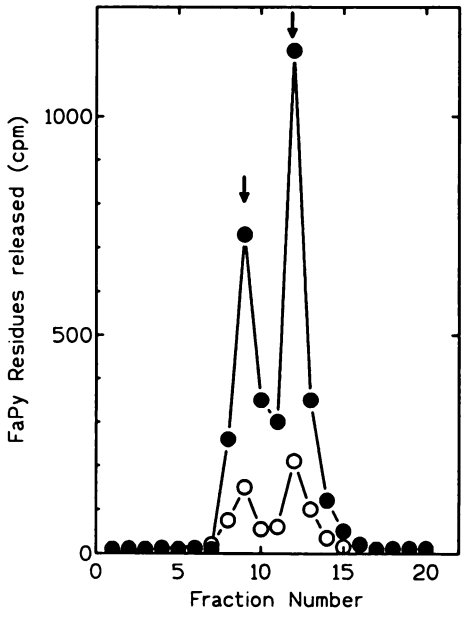

Fig. 1. Excision of FaPy residues from [^{3}H]FaPy-poly[d(G-C)] by CHO cell extracts. Cell extracts (50 μg proteins) from control CHO (○) or CHO-fpg (●) cells were incubated for 30 min at 37°C with the substrate and the release of FaPy residues was analyzed by HPLC. For details, see "Materials and Methods." *Arrows*, positions of the two FaPy rotamers.

QPCR conditions were established to ensure optimal yield while remaining in the exponential phase of the amplification. Under these conditions, the only limiting factor of the amplification is the template DNA; therefore, damage to the DNA will cause a proportional decrease in the amount of the radioactive full-length product, as described (17). These conditions were verified by the addition of a control with one-half of the amount of DNA of one of the untreated cell controls, which resulted in one-half of the amount of products as measured by scintillation counting (data not shown). PCR products were run on a 1.5% agarose gel to ensure that a single product of 884 bp was produced (data not shown). The results after quantification by scintillation counting of the PCR product are shown in Fig. 3. Data are presented as percentage of untreated control for both the parental CHO cell line and the CHO-fpg cells. The parental CHO cell line clearly shows a decrease in incorporation of radiolabel after treatment with thiotepa. The CHO-fpg cell line, on the other hand, sustains a level of damage that does not increase as the dose of thiotepa is increased, suggesting that the Fpg protein may confer a protective effect and that some repair may be occurring over the 3 h of drug exposure.

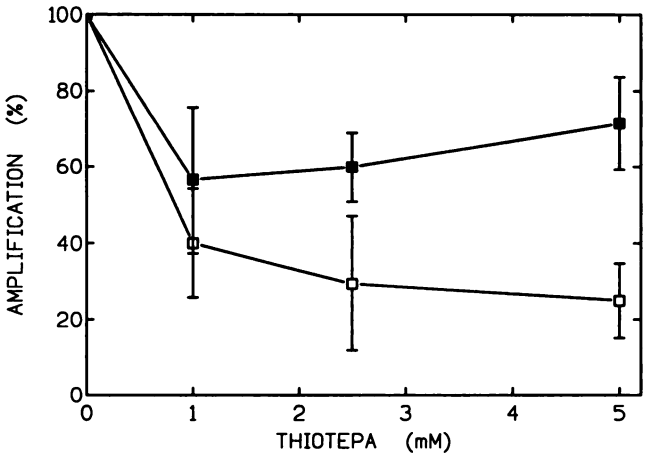

Fig. 3. Quantitation of PCR products obtained from CHO and CHO-fpg cells treated with thiotepa. The values are expressed as percentages of untreated control from parental (□) or fpg-transfected (■) CHO cells. A representative experiment is shown. *Bars*, SD for the triplicate PCR samples in the experiment.

DISCUSSION

The efficiency of drugs used in cancer therapy is limited by tumor resistance, either constitutive or acquired. Different factors are involved in this resistance, but the toxicity of drugs that interfere with DNA is believed to be mostly due to DNA damage and to the repair capacity of the tumor cells.

Two mechanisms have been proposed to explain the activity of thiotepa: either the formation of cross-links within the cellular DNA (3) or the liberation of aziridine (4), which alkylates the DNA, resulting in the formation of N_7-aminoethylguanine residues (4). These residues are unstable and are readily transformed in ring-opened form (aminoethyl-FaPy; Ref. 5). However, the formation of such residues in the DNA of thiotepa-treated cells has never been established.

Ring-opened guanines are repaired in *E. coli*, independently of the substituent, by the Fpg protein (7). Because Fpg-deficient eukaryotic cell lines have not yet been identified, we have overexpressed the bacterial protein in mammalian cells to determine whether this expression resulted in increased cellular resistance to the toxic effect of thiotepa and, therefore, whether ring-opened guanines were formed by this compound.

Our results show that cells expressing the Fpg protein are more resistant to the lethal effect of thiotepa. This suggests that the foreign protein is able to repair some of the damage involved in the killing effect of the drug, and, due to the specificity of the Fpg protein, this strongly suggests that FaPy residues are among the toxic lesions formed by thiotepa in mammalian cells. Expression of the Fpg protein also reduces the thiotepa-induced mutation frequency, suggesting that the drug induces lesions that are mutagenic and Fpg sensitive, *e.g.*, C_8-oxopurines and/or modified adenines (8).

It has been shown that methyl ring-opened guanines are a block to DNA replication (18). If such ring-opened residues are formed by thiotepa in the cells, they should be a block to DNA polymerase and be detected by QPCR. This technique was used previously to detect replication-blocking lesions in the DNA of cells treated with different agents, *e.g.*, UV light or cisplatin (17, 19). Our results show the presence of replication-blocking lesions in the DNA of thiotepa-treated cells and show also that the number of these lesions is lower in cells expressing the Fpg protein. This strongly suggests that the bacterial protein repairs those thiotepa-induced lesions that are a block to the *Taq* DNA polymerase.

The toxicity of thiotepa depends on different factors, especially the presence of oxygen (20) or glutathione (21), and activation by microsomes (3). However, the capacity of the cells to repair DNA damage, especially ring-opened guanines, may play an important role in the cytotoxic action of this drug.

Table 2 *Thiotepa-induced mutations in the hprt locus of CHO and CHO-fpg cells*

Results represent the number of mutants per 10^5 viable cells and are the mean values ± SD of six determinations (confidence level, 68%).

Thiotepa concentration (μM)	No. of 6-thioguanine-resistant colonies for 10^5 viable cells	
	CHO cells	CHO-fpg cells
0	<0.2	<0.2
50	6.0 ± 1.0	2.5 ± 1.5
100	9.5 ± 1.5	6.0 ± 2.0
250	20.0 ± 2.0	14.0 ± 2.0
500	38.0 ± 3.0	24.0 ± 3.0

ACKNOWLEDGMENTS

The authors thank Dr. J. A. Hartley for a critical reading of the manuscript.

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