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Protection of Mammalian Cells against Chemotherapeutic Agents Thiotepa, 1,3-*N*,*N*′-Bis(2-chloroethyl)-*N*-nitrosourea, and Mafosfamide Using the DNA Base Excision Repair Genes Fpg and α-hOgg1: Implications for Protective Gene Therapy Applications

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ABSTRACT

Chemotherapeutic agents used in the treatment of cancer often lead to dose-limiting bone marrow suppression and may initiate secondary leukemia. *N*,*N*′,*N*″-triethylenethiophosphoramide (thiotepa), a polyfunctional alkylating agent, is used in the treatment of breast, ovarian, and bladder carcinomas and is also being tested for efficacy in the treatment of central nervous system tumors. Thiotepa produces ring-opened bases such as formamidopyrimidine and 7-methyl-formamidopyrimidine, which can be recognized and repaired by the formamidopyrimidine glycosylase/AP lyase (Fpg) enzyme of *Escherichia coli*. Using this background information, we have created constructs using the *E. coli fpg* gene along with the functional equivalent human ortholog α-hOgg1. Although protection with the Fpg protein has been previously observed in Chinese hamster ovary cells, we demonstrate significant (100-fold) protection against thiotepa using the *E. coli* Fpg or the human α-hOgg1 cDNA in NIH3T3 cells. We have also observed a 10-fold protection by both the Fpg and α-hOgg1 transgenes against 1,3-*N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) and, to a lesser extent, mafosfamide (2-fold), an active form of the clinical agent cyclophosphamide. These latter two findings are novel and are particularly significant since the added protection was in an O^6-methylguanine-DNA methyltransferase-positive background. These results support our general approach of using DNA base excision repair genes in gene therapy for cellular protection of normal cells during chemotherapy, particularly against the severe myelosuppressive effect of agents such as thiotepa, BCNU, and cyclophosphamide.

Dose-escalation studies using chemotherapeutic agents are being attempted to boost survival rates of both adult and pediatric cancers. Chemotherapeutic alkylating agents continue to play a crucial role in most of these dose-intensified chemotherapy protocols. However, despite the use of myeloid growth factor and stem cell support, myelosuppression continues to be a dose-limiting toxicity of many alkylating agents. A number of years ago we, and several other investigators, began a series of investigations using DNA repair cDNAs, particularly those involved in direct reversal or base excision repair, to protect the bone marrow compartment during dose-intensified chemotherapy (for review, see Limp-Foster and Kelley, 2000). Toward this eventual translational goal, we are systematically investigating which DNA repair enzymes are most effective with clinically used chemotherapeutic agents, particularly focusing on the DNA base excision repair (BER) pathway. One such agent, thiotepa (*N*,*N*′,*N*″-triethylenethiophosphoramide), an alkylating agent that is used in breast cancer and neuroblastoma, as well as colon, lung, gastric, bladder, and ovarian cancers, causes the ring-opening of DNA bases following alkylation at the N^7-position of guanine lesions (Chetsanga and Lindahl, 1979). Thiotepa can be hydrolyzed to aziridine, which results in the depurination, and formation of aminoethyl adducts of guanine and

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ABBREVIATIONS: BER, base excision repair; thiotepa, *N*,*N*′,*N*″-triethylenethiophosphoramide; Fpg, formamidopyrimidine glycosylase; CHO, Chinese hamster ovary; BCNU, 1,3-*N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea; LTR, long-terminal repeat; MGMT, O^6-methylguanine-DNA methyltransferase; α-hOgg1, human 8-oxoguanine DNA glycosylase; AP, apurinic.

adenine (Cohen et al., 1991). N^7-Aminoethyl guanosine becomes unstable and degrades by imidazole ring opening and subsequent depurination. These ring-opened damaged bases, formamidopyrimidines, are repaired by members of the DNA base excision repair pathway, such as the *Escherichia coli fpg* (formamidopyrimidine DNA glycosylase), yeast and human α-hOgg1, and *Drosophila* S3 genes (Fig. 1) (Gill et al., 1996; Deutsch et al., 1997; Karahalil et al., 1998). Previous studies have shown a protective effect of the *E. coli fpg* gene against thiotepa in Chinese hamster ovary (CHO) cells (Gill et al., 1996), and recent studies have identified the types of mutations predominantly caused by thiotepa in mammalian cells (Chen et al., 1999).

Data presented here demonstrate that the human α-hOgg1, as well as the *E. coli* Fpg repair enzymes protect NIH3T3 cells against thiotepa, 1,3-*N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea (BCNU), and mafosfamide, the latter an active agent of cyclophosphamide (clinical drug name Cytoxan). The data presented here also represent the first data in mammalian cells that human α-hOgg1 protects against thiotepa-induced DNA damage to a similar extent as the *E. coli* Fpg protein, and demonstrates that a combined glycosylase/AP lyase, such as Fpg and α-hOgg1, can also protect cells against other clinically relevant chemotherapeutic agents such as BCNU and mafosfamide.

Materials and Methods

Construction of Vectors. The *E. coli fpg* gene was cloned from HB101 *E. coli* cells using polymerase chain reaction primers with *Eco*RI and *Sal*I sites, including 5′ and 3′, respectively (5′-CCG GAA TTC ATG CCT GAA TTA CCC G-3′ and 5′-GGC CGT CGA CAT TAC TTC TGG CAC TGC CGA-3′). The fragment was amplified in an PTC-100 thermocycler (MJ Research, Watertown, MA) at 72°C for 10 min, 94°C for 1 min, 55°C for 1 min hot start, and cycled 35 times through 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min cycle ending with a 72°C elongation phase. The reaction conditions were as suggested in the *Tfl* polymerase (Promega, Madison, WI) protocol. The polymerase chain reaction product and pGEX 4T-1 (Amersham Pharmacia Biotech, Piscataway, NJ) were digested with *Eco*RI and *Sal*I and the purified products ligated using T4 ligase (Life Technologies, Gaithersburg, MD). After confirming the sequence by using the T7 Sequence version 2.0 DNA polymerase (Amersham Pharmacia Biotech, Buckinghamshire, England) as per manufacturer's instructions, the *fpg* fragment was digested with *Eco*RI and *Xho*I and ligated using T4 ligase into MSCV2.1, which was previously digested with *Eco*RI and *Xho*I. MSCV2.1-*fpg* plasmid was purified using Midi Prep (Qiagen, Chatsworth, CA), and 10 μg of plasmid was transfected into GP+E-86 using Lipofectin reagent (Life Technologies) as per manufacturer's instructions. Clones were selected using 1 μg/ml G418 (Life Technologies) and titered using NIH3T3 cells. Virus supernatant was collected from viral clones with titers $>1 \times 10^6$ particles/ml and filtered through 0.45-μm Acrodisc filter (Gelman Sciences, Ann Arbor, MI). Filtered supernatant was incubated overnight with NIH3T3 cells in a solution containing 10 μg/ml polybrene (Sigma, St. Louis, MO). After 36 h the infected cells where selected with 1.0 μg/ml G418 and resistant colonies where grown up and screened by Northern analysis for the retroviral transcript. Colonies were maintained in G418 to maintain selective pressure for the maintenance of the transgene.

Two fragments of α-hOgg1 (Rosenquist et al., 1997) were digested with *Eco*RI and *Bsm*I and ligated together using T4 ligase to form the complete coding sequence of α-hOgg1. The product was digested with *Eco*RI and *Not*I and ligated with T4 ligase into the pCI vector (Promega). Plasmid purified with Midi prep column was cotrans-

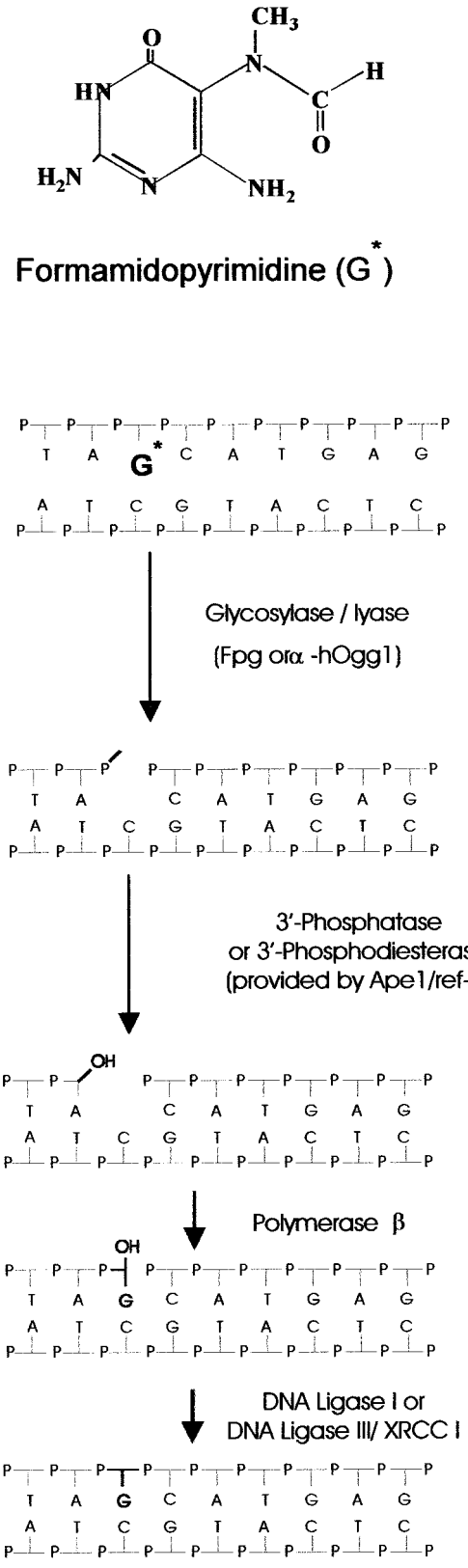

Fig. 1. BER pathway using the combined glycosylase/AP lyase enzymes Fpg or α-hOgg1 in mammalian cells. Agents, such as thiotepa, cause the ring opening of DNA bases following alkylation at the N^7-position of guanine. Aziridine, a hydrolyzation product of thiotepa, causes depurination due to the formation of aminoethyl adducts of guanine and adenine. N^7-Aminoethyl guanine becomes unstable and degrades by imidazole ring opening to form 2,6-diamino-4-hydroxy-5-*N*-methyl formamidopyrimidine (formamidopyrimidine lesions) (Cohen et al., 1991). G*, formamidopyrimidine.

fected with pSK hygromycin resistance plasmid using a 1:10 ratio into NIH3T3 cells. After selection with 200 μg/ml hygromycin (Roche Molecular, Indianapolis, IN), clones were grown up and subjected to Northern analysis for confirmation of transgene transcript production. Hygromycin was kept in the cultures to maintain the presence of the transgene.

Northern Blot Analysis. Total cellular RNA was isolated from cells using RNA STAT-60 (Tel-Test "B" Inc., Friendswood, TX) as per manufacturer's instructions. Total cellular RNA (10 μg) was separated in a 1.2% formamide, agarose gel and transferred onto Hybond-N nylon membrane (Amersham Pharmacia Biotech) using a 10× standard saline citrate solution (1.5 M NaCl, 0.15 M sodium citrate). After the membrane was exposed in a Stratalinker UV crosslinker (Stratagene, La Jolla, CA), the membrane was prehybridized in 10 ml of Hyb-9 solution (Gentra Systems, Minneapolis, MN) for 1 h. Full-length cDNA fragments of each gene were labeled using the DECAprime II DNA labeling kit (Ambion, Austin, TX) and [α-^{32}P]dCTP (Amersham Pharmacia Biotech) as per manufacturer's protocol and 2×10^6 cpm/ml was added to the hybridization solution. Following a 4-h hybridization, the membrane was washed with 0.2× Hyb-9 solution three times for 15 min each, and Hyperfilm MP (Amersham Pharmacia Biotech) was exposed to the membrane overnight.

Cell Proliferation and Colony-Forming Assays. NIH3T3 cells were treated with 0.25% trypsin-EDTA (Life Technologies) and counted using a Coulter counter. Two thousand cells or media alone was aliquoted into each well of a 96-well plate in triplicate and allowed to adhere (approximately 6 h). Thioplex (thiotepa; Immunex, Seattle, WA) was added to a final concentration of 0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, or 1.6 mM for 48 h. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) (20 μl) (CellTiter 96 AQ_{ueous} assay; Promega) was added and allowed to react with the well contents for 4 h after which the optical density was measured at 490 nm. The values were standardized to the media-alone wells.

For the colony-forming assays, NIH3T3 cells were grown in Dulbecco's modified Eagle's medium, 1% penicillin/streptomycin, 10% fetal bovine serum, counted, and seeded on 10-cm^2 tissue culture dishes at a concentrations of 8×10^4 cells/ml (approximately 4×10^5 total cells). After growth overnight at 37°C and 5% CO_2, the cells were treated with drug for 1 h in an incubator and subsequently washed with 1× Dulbecco's PBS. One milliliter of trypsin-EDTA (0.25%:1 mM) was added to each plate and incubated for 1 min. To inhibit further trypsin digestion, 5 ml of media was added to the cells, and a homogeneous cell suspension was produced. An equal mixture of trypan blue stain (0.4%) and cells was analyzed using a hemocytometer. Cells excluding the trypan blue were counted and plated at various concentrations in triplicate on 10-cm^2 tissue culture plates. After 8 days, the colonies were stained with 1% methylene blue in 50% ethanol, washed, and counted.

Statistical Analysis. Experiments were performed in triplicate and repeated at least three times. Statistical analysis was performed using SigmaStat (Jandel Scientific, San Rafael, CA) software package (*t* test and ANOVA) (Hansen et al., 1998).

Results

Construction and Transfection of Mammalian Expression Vectors. To obtain efficient expression in cells, we subcloned the *E. coli* Fpg gene into MSCV2.1 (Fig. 2) from pGEX 4T-1 Fpg (see *Materials and Methods*) using *Eco*RI and *Xho* I. Our choice of using a retroviral construct to express our gene allowed for efficient transduction of genes into various cell types and to provide a source for future experiments in hematopoietic primary cells. MSCV2.1 is derived from the murine embryonic stem cell virus and LN-based retroviral vectors and is used for stable transduction of NIH3T3 cells and hematopoietic progenitor cells (Hansen et al., 1998). We are currently using this construct in bone marrow transplantation experiments in mouse models.

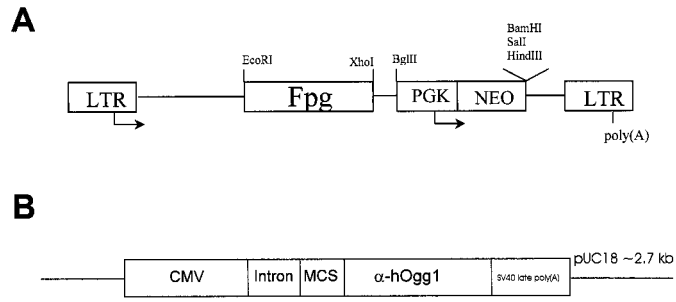

Fig. 2. Mammalian expression constructs for the *E. coli* Fpg and human α-hOgg1 coding regions. A, MSCV2.1 vector containing the Fpg coding region. The expression of the Fpg gene is driven off the retroviral LTR, whereas the phosphoglycerate kinase promoter regulates the neo gene expression. The Fpg coding region was cloned from our pGEX4T-1 construct using *Eco*RI (5′) and *Sal*I (3′). B, pCI vector with the human Ogg1 coding region driven off of the cytomegalovirus promoter.

To produce a mammalian expression vector expressing human 8-oxoguanine DNA glycosylase (α-hOgg1), we obtained two partial clones containing portions of the human α-hOgg1 cDNA. One clone contained all but the first 36 amino acids, and the second clone contained an internal deletion that removed a large portion of the coding sequence. We digested both vectors with *Eco*RI and *Bsm*I, which liberated the correct 5′ sequence fragment from the second plasmid and opened up the first plasmid for insertion of the missing 5′ sequence in the correct reading frame. After ligation and purification of the full-length plasmid we were able to use *Eco*RI and *Not*I to remove the complete human α-hOgg1 coding sequence. The human α-hOgg1 fragment was then inserted into the pCI vector where the cytomegalovirus promoter was replaced with the mouse phosphoglycerate kinase promoter to enhance constitutive expression in the murine system (Fig. 2).

After purifying plasmid from each construct, Lipofectin reagent was used to transduce the Fpg construct into the GP+E-86 retroviral packaging line. Following selection for G418 resistance we collected virus and infected NIH3T3 cells. The α-hOgg1 plasmid construct was cotransfected with pSK hygromycin resistance plasmid directly into NIH3T3 cells. Resistant NIH3T3 cell populations were diluted to isolate single colonies, which were used for subsequent analysis.

Transgene Expression Analysis. Northern blot analysis was performed to determine which resistant colonies contained actively transcribed transgenes and the relative expression level each colony was producing. A representative Northern blot with two transgene-positive clones for NIH3T3-Fpg and NIH3T3-hOgg1, respectively, is shown in Fig. 3, along with NIH3T3 cells alone as a negative control. The 28S and 18S ribosomal bands are shown as a relative size reference. The NIH3T3-Fpg transcript is driven off of the upstream LTR and the polyadenylation site is in the downstream LTR (Fig. 2), which produces a transcript whose length is approximately 4.3 kb (Fig. 3A). α-hOgg1 transgene transcript is approximately 2 kb, which includes the vector's splice donor and splice acceptor sites (Fig. 3B).

Cellular Protection of NIH3T3 Cells Overexpressing Fpg and α-hOgg1 Transgenes. Other investigators have previously demonstrated increased cellular survival to thio-

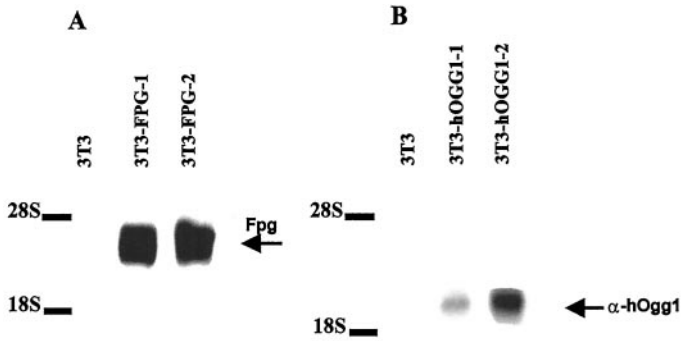

Fig. 3. Northern blot analysis of representative NIH3T3 cells containing either the Fpg or α-hOgg1 transgenes. A, two representative NIH3T3+Fpg clones are shown following Northern blot analysis and detection using radiolabeled Fpg DNA probe. The ribosomal 28S and 18S locations are shown for reference. B, NIH3T3+ α-hOgg1 clones (representative examples) following detection using the radiolabeled α-hOgg1 cDNA as a probe.

tepa by overexpression of Fpg in CHO cells. Initially, we wanted to determine whether this was a general phenomenon and whether we could achieve similar protection in another mammalian cell line, NIH3T3. We analyzed a population of transfected cells that were selected for G418 (neo) resistance to determine whether protection could be obtained on a population of cells, rather than single selected clones. We also picked a number of individual clones and compared them with the populations in the same experiments. The results, shown in Fig. 4A, demonstrate a log order of magnitude of protection with the populations of Fpg-expressing NIH3T3 cells. The individually selected clones that were G418 resistant/Fpg expressing also protected the NIH3T3 cells at all doses of thiotepa above 0.2 mM (Fig. 4). We also selected three 3T3/Fpg clones that, by Northern blot analysis (data not shown), expressed the transgene Fpg at low, medium, and high levels; arbitrary designations with low being barely detectable transcript and high being 10-fold higher level than low. These three clones, although expressing significantly different levels of the Fpg transgene, demonstrated similar protective ability when challenged with thiotepa (Fig. 4B). Although the amount of Fpg RNA levels may not be completely related to biochemical activity, these results demonstrate that in this type of gene therapy use, variation in expression level results in similar protective ability of Fpg. We concluded from these data that above a certain level of Fpg expression, no further enhancement of protective abilities can be achieved. This is, presumably, due to 1) a low amount of this DNA repair activity in mammalian cells, and 2) other rate-limiting steps in the BER pathway downstream of the glycosylase/lyase activity.

We also wanted to determine whether the overexpression of the human ortholog α-hOgg1 would protect mammalian cells to the same extent as the *E. coli* Fpg transgene. As shown in Fig. 5, there was no difference between Fpg and α-hOgg1 in their affording resistance to thiotepa. Although only two clones for Fpg and α-hOgg1 are presented, we have compared a number of other clones and populations with similar results (data not shown).

Fpg and α-hOgg1 Overexpression Protection against BCNU and Mafosfamide. Having demonstrated that Fpg and α-hOgg1 expression does protect against thiotepa in a similar manner, we were interested whether these BER

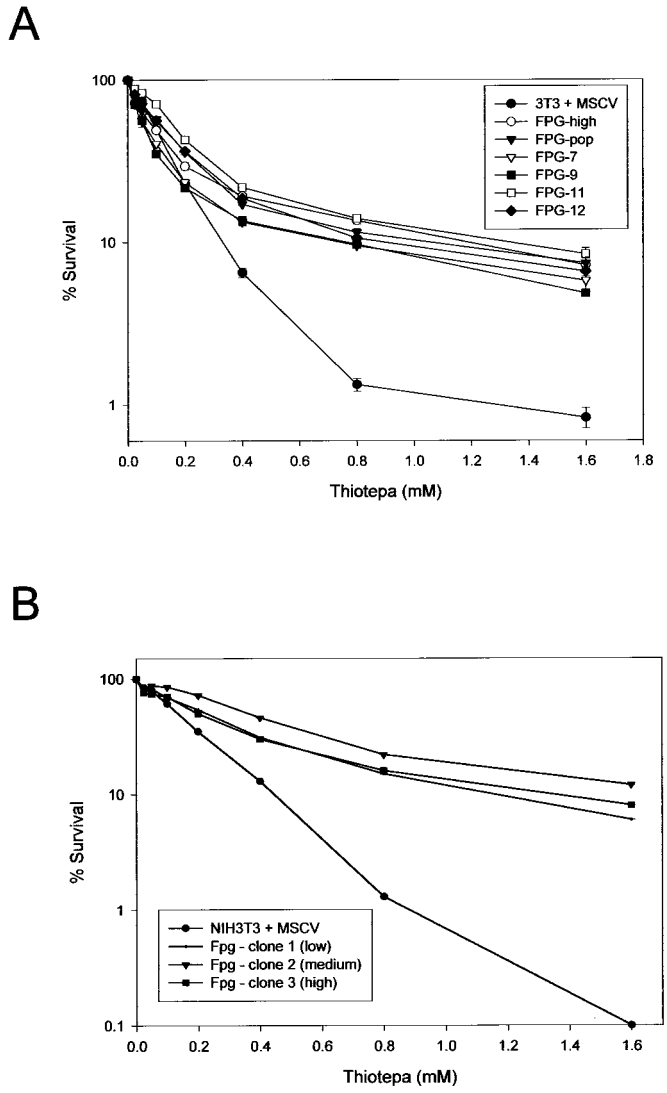

Fig. 4. Cell proliferation assays (MTS) of NIH3T3 clones with vector or Fpg transgenes. A, four individual NIH3T3+Fpg clones (numbers 7, ▽; 9, ■; 11, □; and 12, ♦) and two neo resistant populations of NIH3T3 cells (▼, ○) following Fpg-MSCV transfection were used in cell survival assays with increasing amounts of thiotepa. The individual clones and populations all demonstrated increased protection compared with the NIH3T3 cells containing only the MSCV vector as a control (●). Data are expressed as the mean and standard error of the mean. B, varying expression of Fpg results in similar cytotoxic protection against thiotepa. Three NIH3T3+Fpg containing clones with different amounts of Fpg expression as measured by Northern blot analysis were treated with increasing doses of thiotepa. Data are expressed as the mean and standard error of the mean, which was so small the error bars cannot be detected on the log scale. Experiments were performed in triplicate at least three times.

genes could be used to protect against other clinically used chemotherapeutic alkylating agents. Protection from BCNU-induced toxicity and tumor resistance to this agent has previously been linked to the level of O^6-methylguanine-DNA methyltransferase (MGMT) expression in the tumor. Likewise, cellular toxicity has been linked to the lack of, or decreased, MGMT cellular levels. However, it is clear for the data presented in Fig. 6 that the overexpression of Fpg or α-hOgg1 can also enhance cellular protection against BCNU by 10- to 100-fold in NIH3T3 cells. There was no significant difference between Fpg and α-hOgg1 in the amount of protection (Fig. 6A).

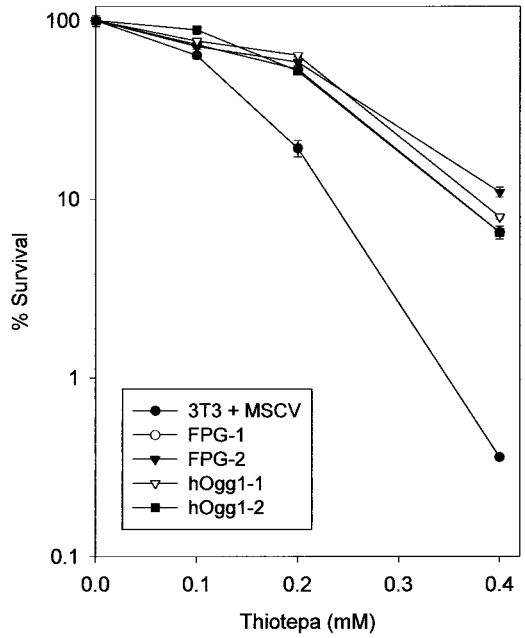

Fig. 5. Colony-forming assays of NIH3T3 cells with both Fpg or α-hOgg1 transgenes and thiotepa. Two clones of the Fpg or α-hOgg1 lines were used in colony-forming assay with increasing doses of thiotepa. Data are expressed as the mean and standard error of the mean for at least three experiments performed in triplicate each time. The control was NIH3T3 cells with the MSCV vector alone (●). Fpg clones (○, ▼) and α-hOgg1 clones (▽, ■) demonstrated similar protective abilities. Other Fpg and α-hOgg1 clones have shown similar results (data not shown).

Finally, as shown in Fig. 6B, cells containing Fpg or α-hOgg1 had statistically significant protection ($p < 0.05$) for the 20 and 30 μM doses of mafosfamide, but not at the lower dose of 10 μM. The protection level at the 20 and 30 μM doses was 2- and 4-fold, respectively, over that of the NIH3T3 cells with vector alone. Again, although only single Fpg and α-hOgg1 clones are shown here, we have observed the same results with a number of other clones for both Fpg and α-hOgg1 (data not shown).

Discussion

DNA BER works through two alternative pathways, designated the short- and long-patch BER (Mitra et al., 1997). Furthermore, the short-patch pathway has been subdivided into two alternative paths (Demple and Harrison, 1994). The pathway of interest for these studies involves a complex glycosylase associated with AP lyase activity (Doetsch and Cunningham, 1990; Mitra et al., 1997) (Fig. 1). Removal of the damaged base and incision of the DNA backbone occurs via a single enzyme. The Fpg and α-hOgg1 DNA glycosylases recognize and initiate repair of 8-oxoguanine and formamidopyrimidine lesions produced by oxidative and alkylation DNA damage, respectively. Further characterization of these enzymes has revealed that the proteins also possesses AP lyase activity removing 5′-terminal deoxyribose phosphate from DNA at an abasic site (Graves et al., 1992), although the Fpg protein has much more efficient AP lyase activity than the α-hOgg1 protein (Zharkov et al., 2000). Therefore, both the *E. coli* Fpg and human α-hOgg1 have at least three activities that include *N*-glycosylase, 3′ and 5′ β-lyase activity, and β-lyase activity for the removal of deoxyribose phosphate. Although previous substrate analysis has identified 8-oxoguanine and formamidopyrimidine as substrates for both Fpg and α-hOgg1 (Karahalil et al., 1998), recent biochemical data have demonstrated that the human α-hOgg1 rate of excision of 7-methyl-formamidopyrimidine to be less than the rate observed with the *E. coli* Fpg enzyme (Asagoshi et al., 2000). Thus, although largely unexplored to date, Fpg glycosylase/AP lyase was presumed to repair alkylation damage in mammalian cells more efficiently than the mammalian functional homologs such as α-hOgg1.

DNA alkylating agents are an important part of most dose-intensified chemotherapy protocols and the BER pathway is clearly involved in the repair of this type of damage (Evans et al., 2000; Hansen and Kelley, 2000; Limp-Foster and Kelley, 2000). This has led us to investigate DNA BER genes that can either enhance protection against drug-induced toxicity, and/or repair damaged sites that may become fixed mutations if not correctly repaired. We also wanted to test whether the Fpg and/or the h-αOgg1 proteins could protect against other agents besides thiotepa, such as BCNU and mafosfamide (cyclophosphamide active agent). The results presented here confirm the protective ability of Fpg against thiotepa-induced lesions in DNA using a retroviral expression construct and demonstrate that the human ortholog α-hOgg1 protein also demonstrated significant protective abilities against thiotepa-induced damage.

Although the Fpg protection against thiotepa was significant (100-fold), we were particularly encouraged that α-hOgg1 gave similar results as the Fpg protein. This is of particular importance since it is more likely that the human counterpart will be useful for human clinical trials given the potential problems of immune response if the *E. coli* Fpg protein was used instead. Furthermore, we were interested to find that the two proteins gave similar protection against the other two drugs we used, BCNU and mafosfamide. This supports our contention that the human α-hOgg1 will be as useful as the Fpg protein and has similar DNA damage recognition and repair capabilities, although we have not directly measured the specific damages in this study.

Recent data demonstrate that the mammalian α-Ogg1 protein has a strong glycosylase, but much weaker AP lyase activity (Zharkov et al., 2000). Therefore, our findings that the human α-hOgg1 protects to a similar degree as the *E. coli* Fpg protein, which does have a strong lyase activity, indicates that some downstream BER proteins may facilitate the lyase activity of α-hOgg1 under our experimental conditions. It has been suggested that α-hOgg1 acts with other members of the BER pathway, namely, the major AP endonuclease (Ape1/ref-1) or β-polymerase to augment its lyase activity or process the AP site hydrolytically (Zharkov et al., 2000). This has been shown to be the case for the human T-G mismatch glycosylase that is dislocated by Ape1/ref-1 (Waters et al., 1999). This could also explain why we see similar protective levels given the differences in expression; downstream enzymes may be rate limiting in mammalian cells. Experiments are currently underway to further explore this possibility and understand the mechanism involved in the protection we observe by expressing downstream BER enzymes (Ape1/ref-1 and/or β-polymerase) to see if we can increase cell survival over just expressing Fpg or α-hOgg1 alone.

We also demonstrated that Fpg and α-hOgg1 protected cells against BCNU, results which were not only somewhat unexpected but also were achieved in cells that are compe-

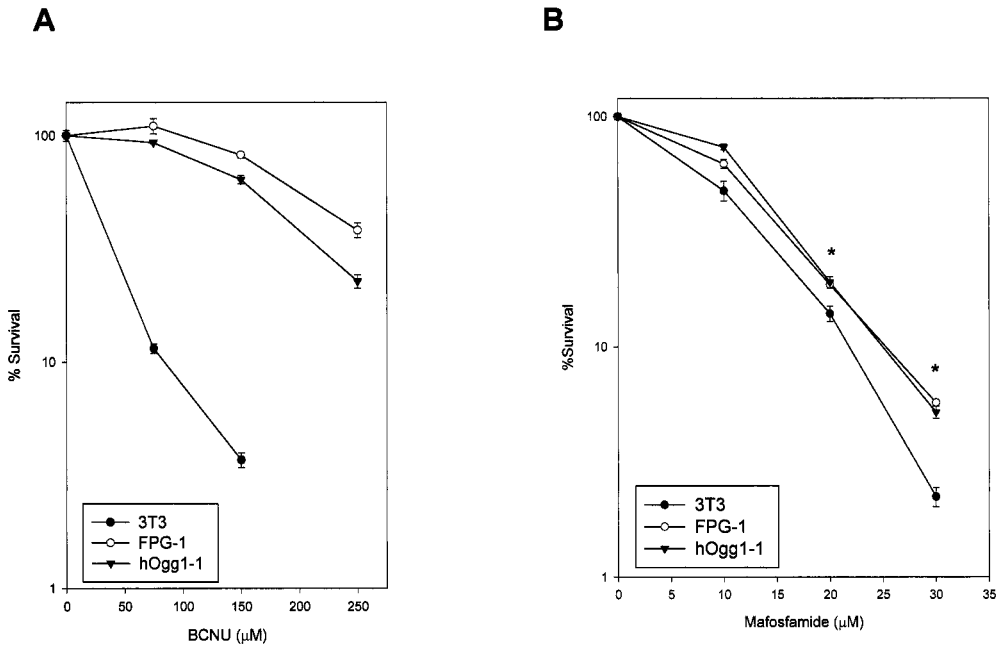

Fig. 6. Protection of NIH3T3 cells with either Fpg or α-hOgg1 against BCNU or mafosfamide. A representative clone of the Fpg or α-hOgg1 lines were used in colony-forming assay with increasing doses of BCNU (A) or mafosfamide (B). Other clones have demonstrated similar results (data not shown). Data are expressed as the mean and standard error of the mean for at least three experiments performed in triplicate each time. The control was NIH3T3 cells with the MSCV vector alone (●). Fpg clone (○) and α-hOgg1 clone (▼) demonstrated similar protective abilities against BCNU (A) or mafosfamide (B). *$p < 0.05$.

tent for MGMT expression. Therefore, this protective ability was in addition to the protective effect of the endogenous MGMT in the NIH3T3 cells. A possible explanation of this finding was found in previous reports demonstrating that BCNU leads to chloroethylamino purine modifications, and that these adducts are unstable, further decomposing into ring-opened nucleotides that are repaired substrates for Fpg and α-hOgg1 (Gombar et al., 1980). Again, these results are encouraging because the protection occurred in NIH3T3 cells that are O^6-methylguanine-DNA methyltransferase-positive, i.e., they have endogenous MGMT present. This also reinforces the fact that BCNU produces other types of DNA adducts and damage besides the O^6-methylguanine adduct, and the other lesions can be cytotoxic and mutagenic if not correctly repaired. This was recently documented in a study demonstrating the production of mutations by agents that produce O^6-methylguanine, even in cells expressing MGMT (Bielas and Heddle, 2000; O'Neill, 2000). Currently, we are testing this hypothesis further in hematopoietic cell lines, such as K562, which are Mer^- (MGMT deficient) and in primary mouse bone marrow stem cells. This strategy will help us to understand how much of the cell killing in hematopoietic cells is due to the cross-linking versus the other DNA-damaging affects in the cells.

Cells containing Fpg and α-hOgg1 were also protected from the cytotoxicity of mafosfamide compared with NIH3T3 cells alone (Fig. 6B). The observed 2-fold protection, although statistically significant ($p < 0.05$), might not appear that impressive compared with the higher degree of protection observed with thiotepa and BCNU. However, studies by other investigators, for example, using overexpressed glutathione-*S*-transferase in cell lines which offered only minor protection (2–3-fold) to cyclophosphamide in the tissue culture cells, similar to what we see, subsequently demonstrated protection in animal models following transduction of glutathione-*S*-transferase to the transplanted bone marrow (Kuga et al., 1997). Therefore, although a fewfold increase in protection in cell lines may not initially appear remarkable, additional animal model studies could reveal a more biologically significant result. Future studies with Fpg and α-hOgg1 in mouse primary progenitor cells and transplanted transduced cells are necessary to establish a similar effect for Fpg- or α-hOgg1-containing cells and are in progress. Furthermore, treatment of cells with different alkylating or oxidative agents such as temazolomide, busulfan, bleomycin, or Adriamycin, or ionizing radiation might demonstrate other possible applications for these DNA repair proteins. This is supported by the recent report demonstrating that when the human α-hOgg1 (same cDNA as used in this study) was targeted to the mitochondria of mammalian cells, enhanced protection of the cells, and the mitochondrial DNA was observed following treatment with the redox-cycling drug menadione (Dobson et al., 2000).

In conclusion, data presented in this manuscript further support the original finding of Fpg expression in mammalian cells affording protection against thiotepa-induced DNA damage (Gill et al., 1996). However, we have expanded upon these findings using the human ortholog α-hOgg1, which appears to provide equivalent protective capacity as Fpg against thiotepa damage. We have also demonstrated that Fpg and α-hOgg1 can substantially protect against BCNU DNA damage, even when endogenous MGMT is present, and also against mafosfamide, although in the latter case not nearly as dramatically. Future studies in animal models and experiments to delineate whether there is a downstream rate-limiting step in the BER pathway are now underway.

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