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Retrovirus-mediated Expression of a DNA Repair Protein in Bone Marrow Protects Hematopoietic Cells from Nitrosourea-induced Toxicity in Vitro and in Vivo

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ABSTRACT

Severe and delayed myelosuppression is a major side effect encountered with the clinical use of nitrosourea-type chemotherapeutic drugs. The DNA repair protein O6-methylguanine DNA methyltransferase (MGMT) has been shown to repair nitrosourea-induced DNA damage. We therefore investigated the effect of expressing MGMT in hematopoietic cells (via retrovirus-mediated gene transfer) on nitrosourea-induced toxicity. A retroviral vector (N2/ZipPGK-MGMT) expressing the human MGMT cDNA from the phosphoglycerate kinase promoter was constructed. Infection of murine bone marrow with the N2/ZipPGK-MGMT retrovirus significantly increased the survival of murine bone marrow-committed progenitor cells following in vitro exposure to N-N′-bis(2-chloroethyl)-N′-nitrosourea (BCNU, carmustine). MGMT gene transfer also protected murine hematopoietic cells in vivo in a murine model of BCNU-induced myelosuppression. The infusion of 4–6 × 10⁶ N2/ZipPGK-MGMT-transduced bone marrow cells into mice every 2 weeks significantly increased peripheral leukocyte counts, platelet counts, and hematocrits compared to infusions of mock-infected bone marrow cells. In addition, bone marrow-committed progenitor cells from some recipient animals demonstrated increased resistance to BCNU in vitro when analyzed 2.5 months after initial treatment. The integration of the N2/ZipPGK-MGMT provirus in the spleen DNA from these animals correlated with the severe bone marrow toxicity induced by CNU compounds in vivo mouse model of sequential BCNU chemotherapy. In this model system, transfection of MGMT-transduced cells partially ameliorated the severe hematotoxicity observed after repetitive BCNU application.

INTRODUCTION

Hematotoxicity is one of the major side effects of chemotherapeutic agents used in the treatment of human malignancies and is the dose-limiting factor in many anticancer treatment protocols. Several genes have been shown to provide protection against the cytotoxic effects of specific chemotherapeutic agents. Theoretically, the transfer and expression of such genes in hematopoietic cells should reduce the bone marrow-related toxic side effects of chemotherapy and allow dose intensification of myelosuppressive agents. As an example, it was shown that retrovirus-mediated transfer of a mutant dihydrofolate reductase cDNA into mouse bone marrow stem cells improves the survival of mice treated subsequently with methotrexate (1–3). Similarly, transfer of the multidrug resistance gene 1 (MDR-1) into murine hematopoietic stem cells has been demonstrated to protect mice against exposure to Taxol (4–6).

The CNUs, such as BCNU, CCNU, or methylcytchexyl nitrosourea (semustine), are a class of alkylating agents that have been used in cancer chemotherapy for over a decade (7). The cytotoxic effects of chloronitrosourea compounds may be alleviated by a number of DNA repair pathways (8). In particular, the mammalian O6-MeG DNA MGMT repairs CNU-induced DNA lesions that if left unrepaired, could produce DNA interstrand cross-links (8). There is a strong suspicion that nitrosourea DNA repair MTases can ameliorate the toxic effects of the chloronitrosourea compounds and that DNA MTases interact with BCNU-treated DNA and can prevent the formation of DNA interstrand cross-links (11–13). In summary, it is clear that O6-MeG DNA MTase proteins can act on chloronitrosourea-induced DNA damage and in so doing can protect mammalian cells from the cytotoxic effects of these agents.

Human and mouse bone marrow cells express extremely low levels of the MGMT DNA repair protein compared to other tissues (14). It seems very likely that this relative MGMT deficiency contributes to the severe bone marrow toxicity induced by CNU compounds in human chemotherapy protocols. We therefore investigated whether increasing the expression of MGMT in hematopoietic cells by retrovirus-mediated gene transfer can decrease the hematotoxicity of CNUs. We report that after transduction with a MGMT-containing recombinant retroviral vector (N2/ZipPGK-MGMT), committed hematopoietic progenitor cells showed increased survival after exposure to BCNU in vitro. In addition, repeated infusions of N2/ZipPGK-MGMT and mock-infected bone marrow cells were compared in an in vivo mouse model of sequential BCNU chemotherapy. In this model system, transfection of MGMT-transduced cells partially ameliorated the severe hematotoxicity observed after repetitive BCNU application.

MATERIALS AND METHODS

Recombinant Vector and Packaging Lines. The human MGMT cDNA was amplified by PCR from the human MGMT cDNA (Ref. 15; a kind gift from Dr. Mutsuo Sekiguchi, Kyushu University, Fukuoka, Japan) with primers incorporating convenient restriction sites and subcloned 3′ to the human PGK promoter in the plasmid pUC-PGK (16). The P3-PGK-MGMT expression cassette was introduced into an empty retroviral construct N2/Zip at a unique Xhol-cloning site (17). N2/Zip contains the 5′ LTR and 3′ LTR and ε+ genome of N2 and the 3′ LTR and genome of Zip retroviral vectors. The structure of the recombinant retroviral vector N2/ZipPGK-MGMT is shown in Fig. 1. In order to be certain that the PCR amplification step did not introduce an inactivating mutation into the MGMT cDNA, the MGMT sequence was reamplified from the retroviral

Received 11/7/94; accepted 4/10/95.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 D. A. W. and L. S. are supported by National Cancer Institute Program Project Grant 5P01 CA 59348-02. L. S. is supported by National Cancer Institute Grant CA55042 and is a recipient of a Burroughs Wellcome Toxicology Scholar Award.

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The abbreviations used are: CNU, chloroethylnitrosourea; BCNU, carmustine; N-N′-bis(2-chloroethyl)-N′-nitrosoureas; CCNU, lomustine, cyclohexylnitrosourea; O6-MeG, O6-methylguanine; MGMT, O6-methylguanine DNA methyltransferase protein; PGK, phosphoglycerate kinase; LTR, long terminal repeat; IL, interleukin; MTase, methyltransferase; rhu, recombinant human.
A/rrnr.v, expected transcripts, b. MGMT activity in various tissues of C57B1/6J mice. Construct expressed active MGMT in E. coli (data not shown). N2/ZipPGK-MGMT retroviral producers were recloned by selection in G-418 and analyzed for the transfer of labeled methyl groups to protein (antiserum kindly provided by Dr. Anthony Pegg, Pennsylvania State University). 

Southern Blot Analysis. High molecular weight DNA was prepared as described previously (25), digested with XhoI (Boehringer Mannheim, Indianapolis, IN), electrophoresed through a 1% agarose gel, and transferred to a nylon filter (Micron Separations, Inc., Westboro, MA). XhoI restriction sites are present in each LTR but not within the proviral structure (Fig. 1). The filter was probed with a random 32P-labeled MGMT cDNA (0.65-kb BamH1/SalI fragment of PGK-MGMT) with the use of a random labeling kit (Boehringer Mannheim). Prehybridization, hybridization, and posthybridization washes were carried out as recommended by the manufacturer. Filters were exposed to X-ray film at −70°C in the presence of a tungsten-intensifying screen.

Retroviral Infection of Bone Marrow Cells. Bone marrow cells were harvested as described previously (23) from the hind limbs of C57B1/6J mice (Jackson Laboratories, Bar Harbor, ME). To target progenitor cells, two modifications of the infection protocol were used: (a) bone marrow cells were harvested 9 days after a single i.p. injection of 5-fluorouracil (150 mg/kg; Solo Pak Laboratories, Franklin Park, IL); and (b) bone marrow was prestimulated for 24 h with a combination of growth factors, including 100 units/ml recombinant murine IL-3 (Pepro Tech, Inc., Rocky Hill, NJ), 100 ng/ml rhuIL-11 (Genetics Institute, Boston, MA), 100 ng/ml recombinant rat stem cell factor, 100 units/ml rhu granulocyte-colony-stimulating factor, and 4 units/ml rh erythropoietin (all from Amgen, Thousand Oaks, CA) in α-MEM-20% FCS supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin. Prestimulated bone marrow cells (3 × 10⁸) were cocultured subsequently on mitomycin C-treated (7.5 µg/ml for 2 h; Bristol-Myers-Squibb, Princeton, NJ) N2/ZipPGK-MGMT producer cells in the presence of growth factors (as above) and 5 µg/ml polybrene. In some experiments prestimulated cells were infected with supernatant from the producer lines without cocultivation. For supernatant infection, 10 ml of virus-containing medium supplemented with fresh growth factors and polybrene was replaced every 12 h for a total of 4 medium changes.

Clonogenic Methylelulocele Assays. Mariner-committed progenitors were assayed as described previously (23). For determining BCNU sensitivity of progenitor cells, 1 × 10⁶ bone marrow cells were incubated in 4 ml α-MEM-20% FCS supplemented with 0.80 μM of BCNU (National Cancer Institute, Drug Synthesis Branch, Bethesda, MD; prepared per manufacturer’s instructions) for 1 h. BCNU-treated cells were washed twice with medium and assayed for surviving progenitor cells in methylcellulose cultures.

Animal Model of BCNU-induced Hematotoxicity. C57B1/6J mice (Jackson Laboratories) were given injections i.p. with 40 mg/kg BCNU weekly starting with day 1 as described previously (24). We continued to treat animals throughout the entire 10 weeks of the experiment since we were attempting to establish maximum doses tolerated for both progenitor transduction protocol (shown here) and stem cell transduction protocols to be utilized in future experiments. On day 3, and thereafter every 2 weeks, animals were infused with 4–6 × 10⁶ N2/ZipPGK-MGMT or mock-infected bone marrow cells via tail vein. Retroviral infections were performed according to the protocol described above. At 2-week intervals (on the day prior to cell infusion), animals were bled by tail vein for determination of peripheral blood leukocyte, platelet counts, and hemocrits as described previously (23). In each experiment 5–10 mice were used per experimental group. In one experiment, animals surviving after 10 weeks were sacrificed by cervical dislocation, and bone marrow and spleen cells were harvested and analyzed for cellularity, progenitor content, and BCNU-resistance of hematopoietic progenitor cells.
was prepared from spleen and bone marrow cells of these animal for Southern blot analysis.

**Statistical Analysis.** Wilcoxon signed rank test was used to analyze in vitro BCNU-resistance data, and Wilcoxon ranked sum test was used to analyze data from in vivo animal experiments.

**RESULTS**

We hypothesized that expression of the human O6-MeG DNA MTase MGMT protein in mouse bone marrow cells would afford mice some extra resistance to the hematotoxic effects of chloro-nitrosourea compounds. The bone marrow of CD-1 mice was shown previously to be extremely deficient in MGMT activity (14) compared to other tissues; in preparation for this study, we confirmed that the bone marrow of C57B1/6J mice was also deficient in MGMT activity (Fig. 1b). Tissue MGMT levels were similar between the CD-1 and C57B1/6J strains of mice except that in the latter, intestine MGMT levels were much lower than those reported for CD-1 (Fig. 1b).

**Construction of Vector and Generation of Producer Lines.** The human PGK promoter used in the N2/Zip retroviral construct has been demonstrated previously to direct high level and stable long-term gene expression in hematopoietic cells in vivo following retrovirus-mediated gene transfer into bone marrow stem and progenitor cells (17, 25–28). We therefore cloned the human MGMT cDNA under the control of the PGK promoter in the same transcripational orientation as from the LTR.

Producer clones for N2/ZipPGK-MGMT were generated by co-transfection with pSV2Neo into the ecotropic GP+E-86-packaging cell line, followed by repeated infection of these transduced clones with virus harvested from transduced GP+envAmm12-producer lines, and analyzed for the production of human MGMT protein with the use of a polyclonal rabbit antiserum that does not cross-react with the mouse MGMT protein. Fourteen cloned transfectants were screened by Western blot analysis. The analysis of seven of these clones is shown in Fig. 2a. Two clones, OMG-9 and OMG-10, produced very high levels of the human MGMT. Indeed, the level of MGMT in these producer clones was even higher than that in Mer+ HeLa cells, which express about 100,000 MGMT molecules/cell (29). In comparison to the parental GP+E-86 cell line, both OMG-9 and OMG-10 demonstrated increased MGMT activity as measured by the transfer of methyl groups from DNA containing O6-MeG to the MGMT protein (Fig. 2b). Moreover, both cell lines acquired substantial resistance to killing by BCNU (OMG-10; data shown in Fig. 2c).

Since high recombinant viral titers are required for transduction of hematopoietic stem and progenitor cells, the OMG-9 and OMG-10 clones were screened for the level of virus production. Dilutions of
supernatant harvested from these clones were used to infect NIH/3T3 cells. Infected cells were analyzed for expression of human MGMT protein by Western blot analysis and for proviral DNA integration by Southern blot analysis. At $10^{-1}$ dilution, NIH/3T3 cells infected with virus from both clones demonstrated MGMT levels similar to that seen in Mer$^+$ HeLa cells (Fig. 2d). Subsequent estimation of the viral titer using Southern blot analysis of infected NIH/3T3 cells demonstrated that OMG-9 had a titer equivalent to about $1 \times 10^3$ virions/ml when compared to a Neo phosphotransferase-containing N2/Zip TkNeo retrovirus (Ref. 17; data not shown).

Infection with PGK-MGMT Protects Hematopoietic Progenitor Cells from BCNU Toxicity in Vitro. To assess the transduction efficiency of primary hematopoietic cells with the N2/ZipPGK-MGMT retroviral vector, murine bone marrow cells were infected by coculture with the producer cell lines OMG-9 and OMG-10. In initial experiments, the survival of bone marrow progenitors achieved after infection with OMG-9 was consistently higher than that achieved with OMG-10 (data not shown). Subsequently, OMG-9 was used for all in vitro and in vivo studies. Fig. 3 shows the BCNU survival curve of bone marrow-committed progenitor cells after coculture infection with OMG-9. A marked increase in the survival of progenitor cells was observed for OMG-9-infected bone marrow cells compared with mock-infected control cells. These experiments were repeated 16 times with marrow harvested from different mice with the use of either cocultivation or supernatant infection protocols. Considerable variations between experiments were detected in the percentage of BCNU-resistant colonies at each dose of BCNU (20 and 40 $\mu$M) for both N2/ZipPGK-MGMT and mock-infected cells (Table 1). However, in all 16 experiments and at both doses of BCNU, N2/ZipPGK-MGMT-infected cells consistently survived better than mock-infected (control) cells. The difference in survival varied from 2 to 66% (median, 16%) for individual experiments and individual BCNU concentrations, and these differences were highly significant ($P < 0.0001$). In three experiments the transduction efficiency for committed progenitor cells was compared with the use of two different infection protocols (coculture or supernatant infection). No significant differences in the BCNU survival curves of committed progenitor cells were detected between these two methods.

Reduction of BCNU-induced Hematopoietic Toxicity following MGMT Gene Transfer in an in Vivo Mouse Model. In order to study the effect of MGMT expression on BCNU toxicity in vivo we developed a murine model of nitrosourea-induced delayed myelosuppression. Weekly administration of BCNU (40 mg/kg, i.p.) over a period of 8–10 weeks produced a profound suppression of all 3 hematopoietic lineages (24). We investigated the protective effect of MGMT-transduced cells on BCNU-induced myelosuppression using this protocol. Four to 6 million MGMT-transduced bone marrow cells were infused every other week, 1 day prior to weekly BCNU administration. More differentiated (and short-lived) hematopoietic progenitor cells were targeted in this transduction protocol (Fig. 4A). Compared with the infusion of mock-infected (control) cells, MGMT-transduced cells provided significant protection in all three hematopoietic lineages. Fig. 4, B–D, shows the results from a representative experiment with six animals/group. Differences were greatest after 6 weeks, reflecting the cumulative effects of multiple BCNU doses. At this point, total leucocyte counts were 1.4 ± 0.4 (SD) versus 0.7 ± 0.2 $\times 10^3$/mm$^3$ (BCNU versus mock), platelet counts were 539 ± 88 versus 308 ± 52 $\times 10^3$/mm$^3$, and hematocrits were 36 ± 1 versus 27 ± 6%. These differences were significant by Wilcoxon rank sum test ($P < 0.05$). After 6 weeks, deaths occurred in both the mock-infected and the MGMT-infected groups, although animals receiving MGMT-transduced cells tended to live longer. Animals in both groups showed signs of progressive BCNU-induced hematotoxicity. Differences in peripheral blood counts were confirmed in two additional experiments.

Demonstration of BCNU Resistance and Molecular Analysis of Animals Infused with Cells Transduced with the N2/ZipPGK MGMT Retrovirus. In one experiment, animals were sacrificed at week 10 (2 weeks after the last infusion of cells), and bone marrow and spleen were analyzed for cellularity, progenitor content, and evidence of proviral integration. In addition, bone marrow from 4 animals receiving MGMT-transduced cells was analyzed for expression of the introduced MGMT by measuring the resistance of harvested bone marrow on exposure to BCNU in vitro. This analysis largely reflects the contribution of the transduced cell population infused 2 weeks prior to analysis, since we have targeted progenitor cells in this transduction protocol. At this time point, cellularity was decreased 2–4-fold, and progenitor content was decreased 10–20-fold, in spleen and bone marrow of BCNU-treated mice (compared to untreated mice). No significant differences in cellularity were noted between animals receiving MGMT versus mock-transduced cells. To our surprise, bone marrow-committed progenitors from only two of the four animals demonstrated increased resistance to BCNU exposure in vitro. However, these two mice were the only animals in which proviral integration could be demonstrated by Southern blot analysis of spleen DNA (Fig. 5). As seen in Fig. 5A, 70 and 88% of the bone marrow-committed progenitor cells from animals 2.4 and 1.5 survived treatment with 40 mM BCNU, compared to 40% in the other 2 animals and untreated animals. In addition, at all BCNU doses examined, increased numbers of committed progenitors survived BCNU exposure (in comparison to controls). Although no proviral integration was

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<th>BCNU (μM)</th>
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<tr>
<td>20</td>
<td>69.7 ± 11.9a</td>
<td>41.2 ± 23.5</td>
<td>28.5 ± 19.3</td>
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<td>40</td>
<td>38.6 ± 21.0</td>
<td>20.4 ± 19.3</td>
<td>18.2 ± 14.5</td>
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a Percentages of colonies resistant to various concentrations of BCNU. Means, ± SD of 16 independent experiments.

Fig. 3. Survival of murine-committed progenitor cells after exposure to BCNU. Murine bone marrow cells infected with N2/ZipPGK-MGMT retrovirus (●) and cells mock-infected (○) were exposed to various concentrations of BCNU for 60 min and thereafter plated in semisolid medium to assay for progenitor-derived colonies, as described in "Materials and Methods."
detectable by Southern blot analysis of DNA obtained from bone marrow, provirus of the expected length of 3.2 kb was detectable at low copy numbers in the DNA from spleens in 3 of 6 analyzed animals (specifically, animals 2.4 and 1.5; Fig. 5B). Therefore, the presence of the provirus in spleen DNA correlated with demonstration of progenitor resistance. In other words, both animals demonstrating increased survival of progenitor cells to BCNU exposure in vitro also demonstrated integration of provirus in the spleen cells, while no integrated provirus was detectable by Southern analysis of DNA obtained from spleens of the two animals demonstrating no increased survival of bone marrow progenitor cells after in vitro exposure to BCNU. The lack of detectable provirus by Southern blot analysis at 10 weeks suggests that the increased peripheral counts demonstrated during the earlier time points of the study may have been due to transduction of short-lived progenitor cells.

DISCUSSION

A wide variety of agents used in the treatment of human malignancies alkylate DNA and cause severe cytotoxicity. The nitrosourea-type drugs, such as BCNU, CCNU, and methyl-CCNU, exert their cytotoxic activity at least in part via alkylation at the O6 position of guanine (30, 31). Expression of proteins that repair this O6-guanine alkylation can protect cells from nitrosourea-induced toxicity. For instance, the E. coli Ada O6-MeG DNA MTase and the human MGMT protein confer BCNU resistance to MTase-deficient (Mer−) human tissue culture cells (15, 32), and transgenic overexpression of human MGMT has been shown to block nitrosourea-induced carcinogenesis in a murine thymic lymphoma model (33).

We have investigated MGMT gene transfer into primary hematopoietic cells, which normally express very low endogenous levels of MGMT, and we demonstrate that retrovirus-mediated expression of MGMT in committed hematopoietic progenitor cells increases the resistance of these cells to BCNU in vitro and in vivo. Protection from BCNU cytotoxicity by retrovirus-mediated gene transfer of MGMT suggests that this approach maybe applicable to cancer therapy.

The studies described here have focused on genetic modification of committed hematopoietic progenitor cells to facilitate the use of this technology in human studies (34). In large animals and humans current retroviral infection protocols allow high gene transfer efficiency into these cells. In contrast, transduction of reconstituting human stem cells has been problematic (35). The transduction protocol described here targets bone marrow harvested 9 days after 5-fluorouracil treatment since the number of committed progenitor cells in murine bone marrow is maximal between days 8–10 after single dosage of this agent (36). In addition, prestimulation of cells with multiple growth factors such as erythropoietin, IL-3, IL-11, stem cell factor, and granulocyte-colony-stimulating factor stimulate proliferation and differentiation of committed progenitor cells.

Recent data from our laboratory (24) and previous experiments by Botnick et al. (37) and Neben et al. (38) have shown that BCNU, even

Fig. 4. Experimental protocol and peripheral blood counts of mice after infusion of N2/ZipPGK-MGMT-infected (or mock-infected) bone marrow (BM) and treatment with weekly injections of BCNU. A, protocol showing timing of BCNU injections and BM cell infusions. d3, day 3. B-D, effect on total WBC counts (B), platelet counts (C), and hematocrits (HCT; D). E86, CD, OMG-9; •. Points, mean; bars, SD. *, significant differences (P < 0.05) by Wilcoxon rank sum test.
in single doses, seriously damages primitive hematopoietic stem cells. Although the data reported here demonstrate increased BCNU resistance of committed hematopoietic progenitor cells both in vitro and in vivo, it is clear that the increase in resistance is relatively small and not prolonged. This may be due to inefficient transduction of the target cells and the short-lived nature of hematopoietic progenitor cells. Maximal reduction of BCNU toxicity in hematopoietic cells in vivo may require targeting hematopoietic cells more primitive than committed progenitors. Studies to examine the effect of stem cell transduction with the N2/ZipPGK-MGMT virus are currently underway in our laboratories. In addition, the NCU compounds alkylate DNA at several sites. The MGMT protein acts primarily on alkyl groups located at the O6 position of guanine. However, it has recently become clear that certain 3-methyladenine DNA glycosylases can also repair CNU-induced DNA damage (36, 39). Thus, another approach to improve the protection of hematopoietic cells from nitrosourea-induced damage may be the simultaneous expression of the MGMT and 3-methyladenine DNA glycosylase repair proteins.

In addition to myelosuppression, renal and pulmonary toxicity are other major clinical side effects of BCNU (40). Severe pulmonary fibrosis has been reported, especially following the use of BCNU (41, 42). We did not detect major pulmonary or renal toxicity in the animal model described here, but these side effects may prohibit dose intensification of nitrosoureas in the human clinical setting, even if the myelosuppression can be controlled adequately. However, some evidence suggests that nonhematopoietic toxicities, particularly in the lung, may be less pronounced with CCNU administration (43). This drug may be more applicable for high dose nitrosourea therapy in the clinical setting. In addition, protection from myelosuppressive effects of nitrosoureas may allow increased use of other myelosuppressive agents in combination with chemotherapy protocols. Alternately, simultaneous genetic alteration of bone marrow and respiratory epithelium may address both hematopoietic and pulmonary toxicity induced by nitrosourea.

In summary, our data show that retrovirus-mediated expression of the DNA repair protein MGMT can protect primary hematopoietic cells from nitrosourea-induced toxicity. This approach may be useful in modulating the myelosuppression associated with the clinical use of these agents. Significant protection from severe myelosuppressive effects of nitrosourea agents might allow dose intensification and/or schedule compression of these agents in future human protocols. In a general sense, dysregulated expression of repair enzymes via gene transfer technology may be an important new approach to cancer therapy.

ACKNOWLEDGMENTS

We thank L. Feng for helpful discussions and Dr. Wei Xiao for constructing the N2/ZipPGK-MGMT vector.

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