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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*[1]

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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 O^6-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 $4–6 \times 10^6$ 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 committed progenitor cell resistance to BCNU. These data suggest that MGMT expression in hematopoietic progenitor and precursor cells protects against nitrosourea-induced toxicity and that gene transfer may prove useful in attempts to reduce nitrosourea-induced myelosuppression in the clinical setting.

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,[4] such as BCNU, CCNU, or methylcyclohexylnitrosourea (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 O^6-MeG DNA MGMT repairs CNU-induced DNA lesions that if left unrepaired, could produce DNA interstrand cross-links (8). That the O^6-MeG DNA repair MTases can ameliorate the toxic effects of the chloronitrosourea compounds was deduced from the following: (*a*) upon exposure of human cells to CNU, those expressing the MGMT protein (*i.e.*, Mer^+) formed fewer DNA interstrand cross-links than those lacking MGMT (*i.e.*, Mer^-; Ref. 9); (*b*) the expression of the *Escherichia coli* Ada DNA MTase protein and the human MGMT protein in human cells confers tremendous resistance to the cytotoxic effects of the CNU compounds; (*c*) MGMT inhibition confers CNU sensitivity (10); and (*d*) it has been demonstrated *in vitro* 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 O^6-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, transfusion 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 PGK-MGMT expression cassette was introduced into an empty retroviral construct N2/Zip at a unique *Xho*I-cloning site (17). N2/Zip contains the 5′ LTR and $\psi+$ 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

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[4] The abbreviations used are: CNU, chloroethylnitrosourea; BCNU, carmustine, *N-N'*-bis(2-chloroethyl)-*N*-nitrosourea; CCNU, lomustine, cyclohexylnitrosourea; O^6-MeG, O^6-methylguanine; MGMT, O^6-methylguanine DNA methyltransferase protein; PGK, phosphoglycerate kinase; LTR, long terminal repeat; IL, interleukin; MTases, methyltransferases; rhu, recombinant human.

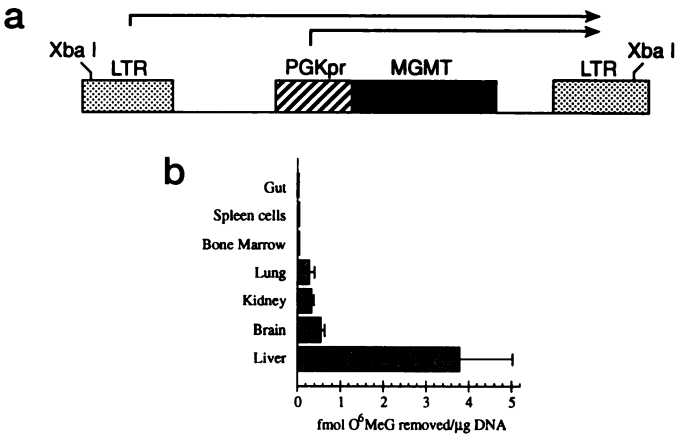

Fig. 1. *a*, structure of the N2/ZipPGK-MGMT retroviral vector. *PGKpr*, human phosphoglycerate kinase promoter. Restriction sites for *Xba*I are shown in 5′ and 3′ LTR. *Arrows*, expected transcripts. *b*, MGMT activity in various tissues of C57Bl/6J mice. *Columns*, mean; *bars*, SD.

construct and cloned under control of the *lac*Z promoter in pUC19. This construct expressed active MGMT in *E. coli* (data not shown). N2/ZipPGK-MGMT was cotransfected with pSV2Neo (10:1) into the ecotropic retrovirus-packaging cell line GP+E-86 (18) and clones resistant to 0.75 mg/ml G-418 (dry powder; GIBCO-BRL, Gaithersburg, MD) were amplified by cross-infection (1) with GP+envAmm12 (19) for 10 days. Subsequently, the ecotropic producers were recloned by selection in G-418 and analyzed for the production of MGMT protein by Western analysis with the use of polyclonal antiserum that recognizes the human but not the murine MGMT protein (20).

GP+E-86 producer cells and the N2/ZipPGK-MGMT producer line clones were maintained in DMEM (GIBCO) supplemented with 10% FCS (Hyclone Laboratories, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin (both from GIBCO). Virus-containing supernatant was collected by adding 10 ml of α-MEM (GIBCO) containing 20% FCS to confluent plates overnight. Harvested medium was filtered through 0.45-μm filters (Gelman Sciences, Ann Arbor, MI) and stored at −80°C until used.

Assays for Retroviral Titers. Cells (1×10^4 NIH/3T3) were seeded on 10-cm tissue culture dishes on day −1 (Falcon Labware, Lincoln Park, NJ) and infected with 2 ml viral supernatant (or dilutions) on day 0 in the presence 7.5 μg/ml polybrene (Aldrich Chemical Co., Milwaukee, WI) at 37°C. Undiluted and 1:10 dilutions of viral supernatant were tested. After 2 h, 8 ml DMEM-10% calf serum were added to the cultures, and the next day the medium was changed. Cells were harvested at confluency, and protein for Western blotting or DNA for Southern blot analysis was prepared. Titer was compared by Southern analysis with a N2/ZipTKNeo retroviral vector (17) of known infectious titer ($1–2 \times 10^5$ G-418-resistant colony-forming units/ml).

Western Blot Analysis of MGMT Expression. MGMT expression from the N2/ZipPGK-MGMT recombinant retrovirus in GP+E-86 producer cells and infected NIH/3T3 cells was evaluated with the use of rabbit polyclonal antiserum (20), which reacts specifically with human but not murine MGMT protein (antiserum kindly provided by Dr. Anthony Pegg, Pennsylvania State University College of Medicine, University Park, PA). Briefly, protein extracts were prepared by sonication, and protein concentration of cleared lysates were determined by Bradford assay. Approximately 100 μg total protein/lane were separated by SDS-PAGE, transferred to nitrocellulose, and probed with a 1:200 dilution of the human MGMT-specific antibody. The blots were developed with the use of an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (1:1000) and the chromogenic substrates nitro blue tetrazolium/bromochloroindolyl phosphate following recommendations of the manufacturer (Bio-Rad Laboratories, Richmond, CA).

Determination of O^6-Methylguanine-DNA Methyltransferase Activity Levels in Mouse Tissues. Various tissues from C57Bl/6J were analyzed to determine the levels of O^6-methylguanine-DNA methyltransferase activity. Tissues were prepared essentially as described by Gerson *et al.* (14). Briefly, following surgical removal, tissues were placed in an equal volume of methyltransferase buffer [50 mM HEPES (pH 7.8)-10 mM DTT-1 mM EDTA-5% glycerol) and stored frozen (−80°C) prior to processing. Tissues were thawed on ice, two additional volumes of buffer were added, and then tissues were disrupted on ice with the use of dounce homogenization. Disruption was completed by sonication, and the concentration of DNA in the crude lysate was determined with the use of Hoescht dye 33258 fluorometry as described (14). The lysate was then cleared by centrifugation ($12,000 \times g$; 5 min; 4°C), and the concentration of protein in cleared lysate was determined by Bradford assay. Aliquots were frozen in liquid nitrogen and stored at −80°C prior to enzyme activity determination. O^6-methylguanine-DNA methyltransferase activity was determined with the use of the rapid assay of Margison *et al.* (21). The methylated DNA substrate was prepared by reacting *Micrococcus luteus* DNA (Sigma Chemical Co., St. Louis, MO) with [^{3}H]methylnitrosourea (18 Ci/mmol; Amersham, Amersham, United Kingdom) essentially as described (22). The specific activity of the substrate was approximately 140 cpm/μg. The transfer of labeled methyl groups to protein was determined for all tissues under nonsaturating conditions.

Southern Blot Analysis. High molecular weight DNA was prepared as described previously (25), digested with *Xba*I (Boehringer Mannheim, Indianapolis, IN), electrophoresed through a 1% agarose gel, and transferred to a nylon filter (Micron Separations, Inc., Westboro, MA). *Xba*I restriction sites are present in each LTR but not within the proviral structure (Fig. 1). The filter was probed with a random ^{32}P-labeled MGMT cDNA (0.65-kb *Bam*HI/*Sal*I 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 C57Bl/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 rhu 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 (3×10^6) were cocultured subsequently on mitomycin C-treated (7.5 μg/ml for 2 h; Bristol-Myers-Squibb, Princeton, NJ) N2/ZipPGK-MGMT producer clones 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 Methylcellulose Assays. Murine-committed progenitors were assayed as described previously (23). For determining BCNU sensitivity of progenitor cells, 1×10^6 bone marrow cells were incubated in 4 ml α-MEM/20% FCS 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. C57Bl/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 $4–6 \times 10^6$ 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 hematocrits 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. DNA

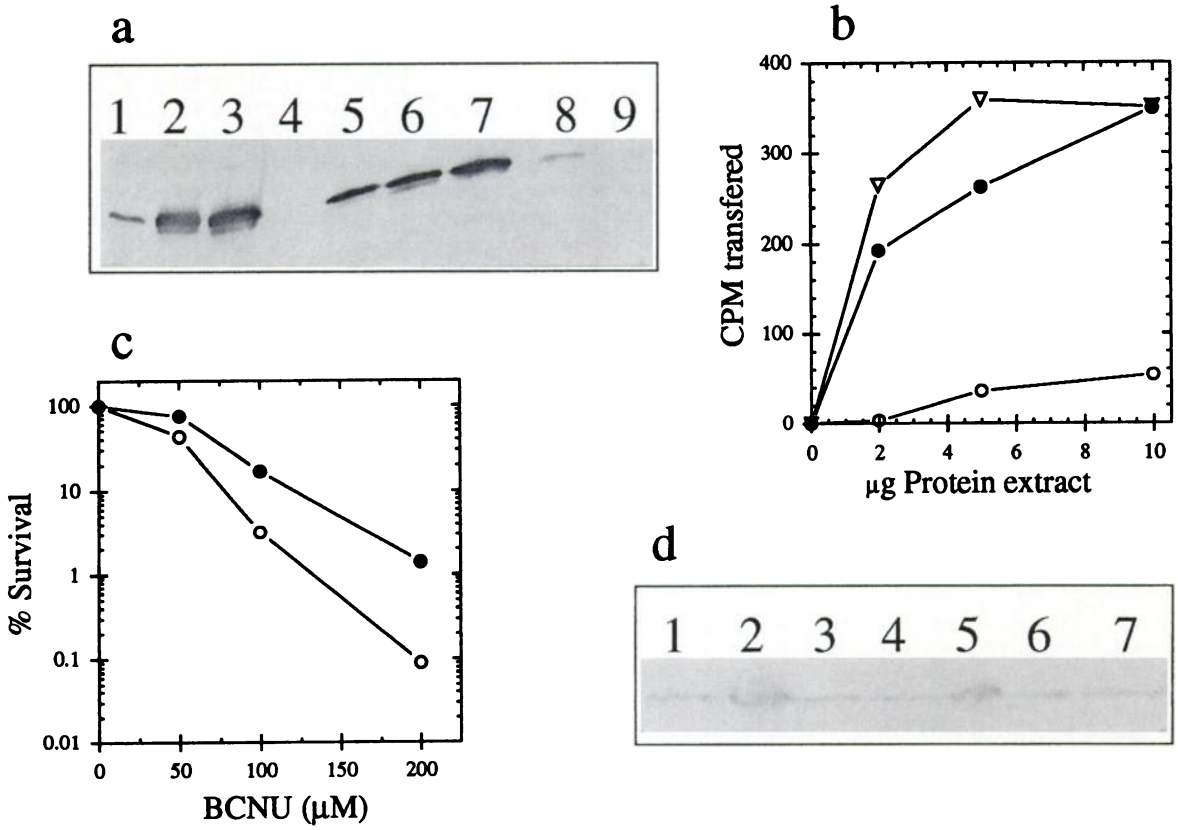

Fig. 2. Production of N2/ZipPGK-MGMT retroviral producer cell lines. *a*, Western blot analysis of GP+E-86 retroviral producer cells and selected transfectants. A number of G-418-resistant GP+E-86 primary transfectants (*Lanes 1-7*) were screened for expression of the human MGMT protein with the use of human MGMT-specific antibodies. Mer^+ HeLa cells (*Lane 8*) and nontransfected GP+E-86 cells (*Lane 9*), positive and negative controls, respectively; *Lanes 1-7*, 7 independent transfectants from a total of 14 that were screened. Transfectants 9 (OMG-9, *Lane 3*) and 10 (OMG-10, *Lane 2*), which produced the highest levels of human MGMT protein of those transfectants examined, were chosen for further analysis. *b*, O^6-methylguanine-DNA-methyltransferase levels for GP+E-86 cells and selected transfectants. O^6-methylguanine-DNA-methyltransferase activity levels were determined for the GP+E-86 packaging cell line (○), OMG-9 (●), and OMG-10 (▽), as described in "Materials and Methods." *c*, BCNU-induced killing of GP+E-86 (○) and clone 9 (●). *d*, Western blot analysis of extracts from NIH/3T3 cells infected with N2/ZipPGK-MGMT viral supernatant. NIH/3T3 cells were infected with viral supernatant, and at confluence, cell extracts were prepared and probed with human MGMT-specific antibodies. *Lanes 3* and *4*, NIH/3T3 cells infected with a 10^0 or 10^{-1} dilution, respectively, of N2/ZipPGK-MGMT supernatant from clone 9. *Lanes 6* and *7*, NIH/3T3 cells infected with a 10^0 or 10^{-1} dilution, respectively, of N2/ZipPGK-MGMT retroviral supernatant from clone 10. Extracts from clones 9 (*Lane 2*) and 10 (*Lane 5*) and HeLa Mer^+ cells (*Lane 1*) are included as positive controls. Extracts from NIH/3T3 cells not infected with recombinant retrovirus display no detectable signal with these antibodies at these dilutions (data not shown).

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 O^6-MeG DNA MTase MGMT protein in mouse bone marrow cells would afford mice some extra resistance to the hematotoxic effects of chloronitrosourea 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 C57Bl/6J mice was also deficient in MGMT activity (Fig. 1*b*). Tissue MGMT levels were similar between the CD-1 and C57Bl/6J strains of mice except that in the latter, intestine MGMT levels were much lower than those reported for CD-1 (Fig. 1*b*).

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 transcriptional 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. 2*a*. 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 O^6-MeG to the MGMT protein (Fig. 2*b*). Moreover, both cell lines acquired substantial resistance to killing by BCNU (OMG-10; data shown in Fig. 2*c*).

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. 2*d*). 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 1×10^5 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 μM) 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.

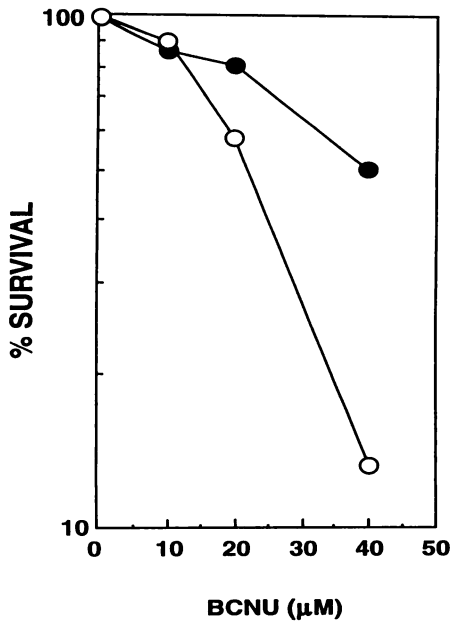

Fig. 3. Survival of murine-committed progenitor cells after exposure to BCNU. Murine bone marrow cells infected with N2/ZipPGK-MGMT retrovirus (●) and cells mock-infected (○) were exposed to various concentrations of BCNU for 60 min and thereafter plated in semisolid medium to assay for progenitor-derived colonies, as described in "Materials and Methods."

Table 1 *Generation of BCNU-resistant hematopoietic progenitor cells*

BCNU	PGK-MGMT	Control	Δ
20 μM	69.7 ± 11.9[a]	41.2 ± 23.5	28.6 ± 19.3
40 μM	38.6 ± 21.0	20.4 ± 19.3	18.2 ± 14.5

[a] Percentages of colonies resistant to various concentrations of BCNU. Means, ± SD of 16 independent experiments.

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. 4*A*). 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 leukocyte counts were 1.4 ± 0.4 (SD) *versus* 0.7 ± 0.2 × 10^3/mm^3 (BCNU *versus* mock), platelet counts were 539 ± 88 *versus* 308 ± 62 × 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. 5*A*, 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

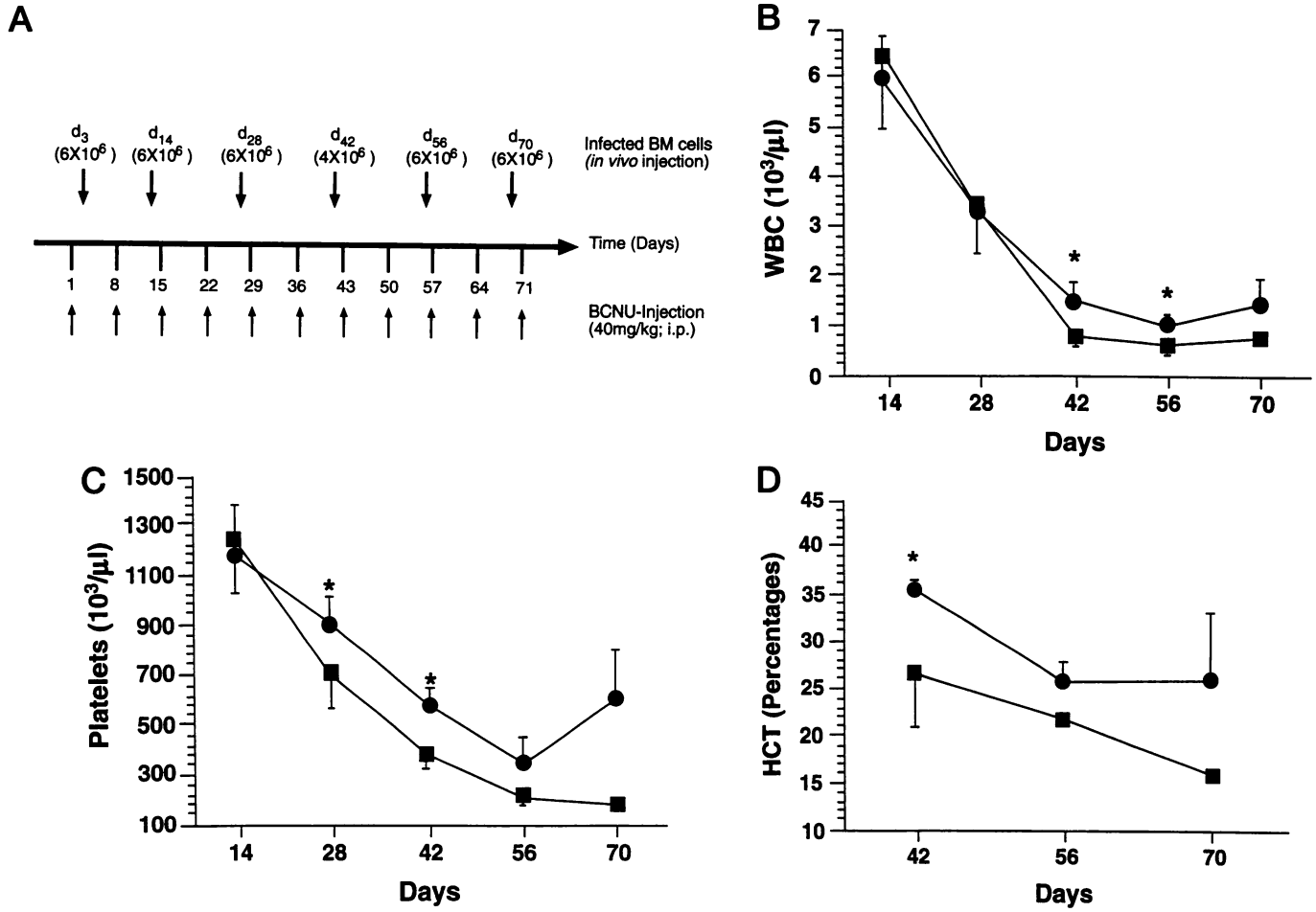

Fig. 4. Experimental protocol and peripheral blood counts of mice after infusion of N2/ZipPGK-MGMT-infected (or mock-infected) bone marrow (*BM*) and treatment with weekly injections of BCNU. *A*, protocol showing timing of BCNU injections and BM cell infusions. d_3, day 3. *B-D*, effect on total WBC counts (*B*), platelet counts (*C*), and hematocrits (*HCT*; *D*). E86, ■; OMG-9, ●. *Points*, mean; *bars*, SD. *, significant differences ($P < 0.05$) by Wilcoxon rank sum test.

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. 5*B*). 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 O^6 position of guanine (30, 31). Expression of proteins that repair this O^6-guanine alkylation can protect cells from nitrosourea-induced toxicity. For instance, the *E. coli* Ada O^6-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

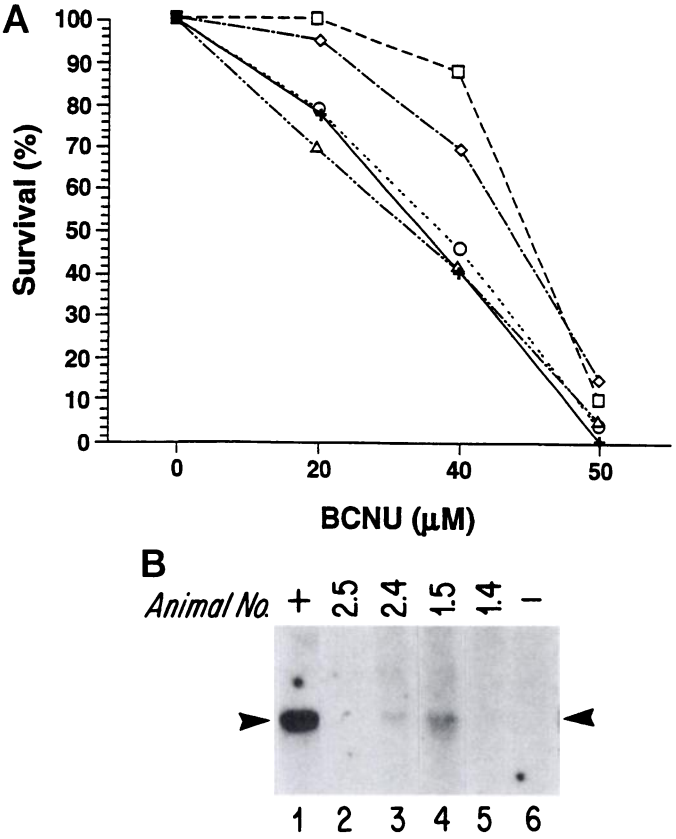

Fig. 5. BCNU resistance of bone marrow progenitor cells harvested from transplanted animals (*A*) and Southern blot analysis of spleen DNA of animals receiving MGMT-transduced cells (*B*). *A*, data for four animals (animal 1.4, 1.5, 2.4, and 2.5) receiving MGMT-transduced cells and one normal animal. +, normal animal; ○, animal 1.2; ◇, animal 1.5; □, animal 2.4; △, animal 2.5]. *B*, *Lane 1*, NIH/3T3 infected with PGK-MGMT (clone 9; positive control); *Lanes 2-5*, spleen cells from animals receiving N2/ZipPGK-MGMT-transduced bone marrow infusions (correlating to *A* as follows: *Lane 2*, animal 2.5; *Lane 3*, animal 2.4; *Lane 4*, animal 1.5; *Lane 5*, animal 1.4) and one negative (−) control animal (*Lane 6*).

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 under way in our laboratories. In addition, the CNU compounds alkylate DNA at several sites. The MGMT protein acts primarily on alkyl groups located at the O^6 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.

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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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