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Retroviral transduction of a mutant methylguanine DNA methyltransferase gene into human CD34 cells confers resistance to O^6-benzylguanine plus 1,3-bis(2-chloroethyl)-1-nitrosourea

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ABSTRACT **Human CD34 cells express low levels of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) and are sensitive to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Gene transfer of the AGT gene, methylguanine DNA methyltransferase (*MGMT*), results in only modest BCNU resistance. Recently, an AGT inhibitor, O^6-benzylguanine (BG), entered clinical trials. In preclinical studies, BG potentiated the cytotoxic effect of BCNU in tumors but increased toxicity to normal CD34 cells. We transferred a mutant *MGMT* containing a glycine-to-alanine mutation at position 156, resulting in marked resistance to BG, into Chinese hamster cells; the K562 cell line and human CD34 cells used the retroviral backbone MFG. In each instance, cells expressed increased AGT and were much more resistant to the combination of BG and BCNU than the parental cells or cells transduced with wild-type *MGMT*. Furthermore, the transduction efficiency in human CD34 cells was in excess of 70%, and the proportion of CD34 transduced cells resistant to the combination was >30%. Thus, retroviral-mediated transduction of a mutant *MGMT* into CD34 cells appears to be an effective way to induce selective resistance to a drug combination designed to overcome a significant resistance mechanism to nitrosoureas in tumors.**

Transfer of a drug resistance gene such as dihydrofolate reductase (1, 2), multiple drug resistance-1 (3–5), and aldehyde dehydrogenase (6) into hematopoietic progenitors is a proposed method to protect the bone marrow from the toxic effects of chemotherapeutic agents, improving tolerance to anticancer agents, and perhaps providing a means for dominant selection of a second therapeutic gene. We (7, 8) and others (9, 10) have recently advocated the use of methylguanine DNA methyltransferase gene (*MGMT*), the gene that encodes the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT), in this setting for a number of reasons. First, AGT confers resistance to multiple alkylating agents including nitrosoureas, dacarbazine, temozolomide, and procarbazine. Second, hematopoietic cells and in particular CD34 hematopoietic progenitors contain low levels of this protein (11, 12), explaining the common finding of dose-limiting, cumulative myelosuppression. Third, there are clear animal and clinical examples of delayed hematopoietic toxicity with the use of these agents including marrow hypoplasia and secondary leukemias (10, 13), suggesting that the sensitivity to nitrosoureas and related compounds extends to early hematopoietic progenitors.

The mechanism of action of the alkyltransferase is unique among DNA repair enzymes. The protein serves as the acceptor of DNA-alkylations at the O^6 position of guanine, the site of one of the most cytotoxic lesions formed by both chloroethylating and methylating agents (14, 15). Repair proceeds by covalent transfer of the adduct to the active site of the protein, a "suicide" process. Cytotoxicity of unrepaired O^6-chloroethylguanine lesions is due to intramolecular rearrangement to O^6-N^1-ethanoguanine followed by interstrand crosslink formation (16), whereas that of the methylating agents is due to recognition of the O^6-methylguanine·cytosine or O^6-methylguanine·thymine (formed after replication) base mispair by the mismatch repair complex and induction of aberrant repair processes leading to multiple DNA strand breaks (17).

We have recently shown that overexpression of *MGMT* protects hematopoietic cells from the toxic effects of nitrosoureas both *in vitro* and *in vivo*. MGMT overexpression in thymic T cells of the mouse prevents methylnitrosourea-induced "secondary" T-cell lymphoma/leukemias (18) and results in rapid loss of the offending O^6-methylguanine DNA adduct from cortical T-lymphocytes (19, 20), the target for the induction of the lymphoma/leukemias. In retroviral gene transfer studies, murine bone marrow cells transduced with human *MGMT* showed 40-fold overexpression of AGT and an increase in 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) resistance in hematopoietic progenitors at least 37 weeks after transplantation into isogeneic mice (7). BCNU treatment of transplanted mice increased the proportion of hematopoietic progenitors carrying the provirus and the mean level of expression of human *MGMT* (21). Likewise, Maze *et al.* (10) showed a survival advantage to mice transplanted with *MGMT*-transduced marrow cells and repeatedly exposed to BCNU. Finally, *MGMT* gene transfer into human CD34 cells resulted in a slight increase in clonogenic survival *in vitro* at high concentrations of BCNU (8).

Although *MGMT* overexpression increases BCNU resistance in normal murine and human hematopoietic cells, the effect has been quite modest, raising questions about its therapeutic utility. Two recent advances have shifted our approach toward therapeutic transfer of *MGMT* into human CD34 cells. First, O^6-benzylguanine (BG) was described as a potent inactivator of the AGT capable of enhancing the antitumor efficacy of BCNU in cell lines and in human tumor xenografts (14, 22–25). Since many tumors express high levels of AGT and are resistant to BCNU and related compounds, BG represents a significant advance in the approach to biochemical modulation of tumor drug resistance (25). Not

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Abbreviations: BG, O^6-benzylguanine; AGT, O^6-alkylguanine-DNA alkyltransferase protein; *MGMT*, methylguanine DNA methyltransferase gene; wt*MGMT*, wild-type *MGMT*; Δ*MGMT*, G156A mutant *MGMT*; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL, interleukin.

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surprisingly, in preclinical toxicology, myelosuppression was dose limiting with BG and BCNU (26). In early phase 1 trials with BG alone, we have observed significant inhibition of the alkyltransferase in human lymphocyte and tumor samples without toxicity (27); however, it appears likely that as the dose of BCNU is increased, myelosuppression will be dose limiting.

Second, a number of AGT point mutations that have been characterized retain DNA repair activity but are remarkably resistant to inactivation by BG. One of these, the glycine-to-alanine mutation at position 156 (G156A) in the human protein results in 240-fold resistance to BG (28). For this reason, we engineered the MFG retroviral vector to contain the G156A mutant *MGMT* (Δ*MGMT*) and studied its ability to confer resistance to the combination of BG and BCNU in human CD34 cells. Transduced cells are remarkably resistant to this combination compared with untransduced cells or cells transduced with wild-type *MGMT* (wt*MGMT*). Thus, our results support the potential therapeutic use of the Δ*MGMT in vivo* to protect hematopoietic cells from the combination of BG and BCNU.

METHODS

Retroviral Vectors. Retroviral vectors pMFG-wt*MGMT* and pMFG-Δ*MGMT* were constructed by inserting the wild-type and G156A mutant human *MGMT* cDNA coding sequences, respectively, into the unique *Nco*I and *Bam*HI restriction sites of pMFG (Paul Robbins, University of Pittsburgh; 29). The *Nco*I containing the ATG start codon and *Bam*HI sites at the 5′ and 3′ termini of human *MGMT* cDNA sequence, respectively, were generated by PCR amplification with primers 5′-p, 5′-CTTGGAACCATGGACAAGGATTGTGAAA-3′; and 3′-p, 5′-CTTAGGATCCCATCCGATGCAGTGTTACACG-3′. Δ*MGMT* cDNA was generated by site-directed PCR mutagenesis in two steps using oligonucleotides containing GCC in place of GGC at codon 156. Sequences were confirmed by the dideoxynucleotide chain termination method (fmol DNA sequencing system, Promega).

Transfection of the Vector Constructs into CHO Cells. A total of 6 μg of each plasmid (pMFG-wt*MGMT* or pMFG-Δ*MGMT*) was cotransfected into 1.8×10^6 CHO cells with 0.6 μg of pSV2neo plasmid DNA with LipofectAMINE (GIBCO/BRL), selected in G418 (1 g/liter), and screened for AGT activity.

Virus-Producing Cells. pMFG-wt*MGMT* or pMFG-Δ*MGMT* and pSV2neo DNA were cotransfected into the packaging line GP + E86, followed by viral supernatant infection of the amphotropic cell line GP + *env*Am12 (30) (Arthur Bank, Columbia University). To increase titer, a supernatant "ping-pong" method was used (31). The amphotropic ΨCRIP MFG-*lacZ* cell line was from Paul Robbins. Titer was estimated from supernatants collected after six daily media changes (7) by infecting 1×10^5 K562 cells (as described below) with limiting dilutions of viral supernatant.

K562 Transduction. The human chronic myelogenous leukemia cell line K562 was retrovirally transduced as described (7). Briefly, wt*MGMT* and Δ*MGMT* producers were treated with 10 μg/ml mitomycin C and replated. Twenty-four hours later, K562 cells were added in the presence of human interleukin 3 (IL-3) (100 units/ml), human granulocyte–macrophage colony-stimulating factor (GM-CSF) (200 units/ml), and polybrene (8 μg/ml) and were collected after a 48-hr coculture.

CD34 Transduction. Peripheral blood mononuclear cells were obtained by apheresis from adult patients treated with cyclophosphamide and/or granulocyte-CSF under an Institutional Review Board-approved protocol (32). CD34 progenitors were isolated using the Ceprate LC Stem Cell Concentrator (Shelly Helmfeld, CellPro, Bothell, WA), followed by an incubation with biotinylated anti-CD34 antibody, passage over an avidin column, and elution by gentle agitation. Recovered cells had an average purity of 57%. For coculture infection, fresh CD34 cells (1×10^5 cells/ml) were cultured for 96 hr in Iscove's modified Dulbecco's medium (GIBCO/BRL) containing 20% heat-inactivated fetal calf serum and supplemented with human stem cell factor (100 ng/ml, Amgen Biologicals), IL-3 (100 units/ml) and IL-6 (100 units/ml) (both from Sandoz Pharmaceutical), and protamine sulfate (4 μg/ml, Sigma) and then were cultured with MFG-Δ*MGMT*, MFG-wt*MGMT*, or MFG-*lacZ* producers. At 48 hr, half of the medium was replaced. For retroviral supernatant infection, CD34 cells were prepared as above and cultured on human bone marrow stroma initiated as follows. Bone marrow mononuclear cells were cultured in myeloid long-term culture medium (Stem Cell Technologies, Vancouver, B.C., Canada) at 2×10^6 cells/ml for 3 days at 37°C/5% CO_2 and transferred to 33°C/5% CO_2, passaged once, and irradiated with 15 Gy 24 hr prior to use. Δ*MGMT* or *lacZ* retroviral supernatant collected from confluent producer cells as described (7) was changed at 24-hr intervals and cultured with CD34 cells as described above. After 72 hr, cells were removed from the stromal layer with cell dissociation buffer (GIBCO/BRL).

***In Vitro* BG/BCNU Treatment.** Cells were resuspended in serum-free medium containing 100 units/ml GM-CSF with or without 10 μM BG and incubated at 37°C for 1 hr (12). BCNU was added to the cells for 2 hr (7), after which cells were incubated for 7–10 days in methylcellulose (Stem Cell Technologies) containing either stem cell factor, IL-3, hemin, erythropoietin, and GM-CSF (8) (for CD34 cells) or GM-CSF and IL-3 (for K562 cells) in triplicate, with or without 5 μM BG.

Immunoassay. Cytospin preparations were stained for AGT using the monoclonal antibody mT3.1 (D. Bigner, Duke University) and biotin/avidin horseradish peroxidase system (Vector Laboratories). Western blots were done as described (33), and the bound antibody was detected by the chemiluminescence ECL kit (Amersham). AGT activity was correlated to densitometric band intensity using *MGMT*-transduced K562 cells (7).

AGT Assay. AGT activity was measured as [^{3}H]methyl groups removed from [^{3}H]O^6-methylguanine present in [^{3}H]methylnitrosourea-treated alkylated calf thymus DNA. The alkylated [^{3}H-methyl]O^6-methylguanine and N^7-methylguanine bases were separated by HPLC and quantified by liquid scintillation. AGT activity was expressed as fmol O^6-methylguanine removed per μg of DNA (34).

PCR Provirus Analysis. DNA was isolated from single colonies as described (7). A 152-bp human *MGMT* fragment that amplifies only the provirus and not endogenous sequences and a 295-bp human dystrophin fragment were amplified and separated by 2% agarose gel.

Reverse Transcription–PCR Analysis. RNA was prepared from 5 to 10 methylcellulose colonies as described (7). In the presence of reverse transcriptase, a 497-bp fragment was amplified using the sense primer (5′-TGGTACCTCACCCTTACCGAGTC-3′) containing sequences of the MFG proviral backbone and the antisense primer (5′-ACACCTGTCTGGTGAACGACTCT-3′) specific to human *MGMT*.

Helper Virus Assay. After retroviral supernatant infection of K562 and NIH 3T3 cells, supernatants were collected and cultured with fresh NIH 3T3 cells or NIH *lac* cells. The former were analyzed for the presence of proviral sequences by PCR. The latter were analyzed for transmission of *lac* plus virus into naive NIH 3T3 cells. Replication-competent virus was not detected in supernatants tested by either assay.

RESULTS

Expression of AGT and BG Resistance in CHO Cells. ΔAGT protein levels and activity was compared with wtAGT in transfected CHO cells by enzyme assay and Western blot analysis. AGT activity was 3.5 fmol/μg DNA in Δ*MGMT*-transfected CHO cells compared with 84 fmol/μg DNA in

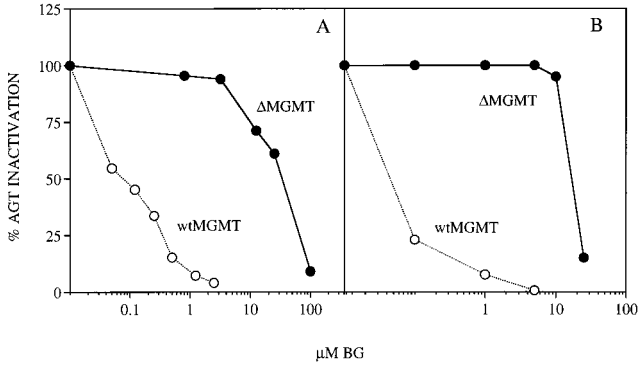

FIG. 1. BG inactivation of wtAGT and ΔAGT. wt*MGMT* or Δ*MGMT* transfected CHO (*A*) or transduced K562 (*B*) cells were exposed to BG for 1 hr. Cells were harvested immediately, and AGT activity in protein extracts was determined by biochemical assay.

wt*MGMT* transfectants, providing independent confirmation that the G156A mutant AGT protein is functional in mammalian cells (35). In CHO cells the EC_{50} for ΔAGT inhibition was ≈30 μM BG compared with <0.1 μM BG for wtAGT (Fig. 1*A*).

Titer. MFG-Δ*MGMT* titer from serial dilutions of Am-12 supernatant was estimated by immunohistochemical detection of infected K562 cells (Fig. 2). A clone with a titer of 5×10^5 AGT-positive infectious particles per ml was used for further experiments. Of note, ΔAGT staining was nuclear, indicating that the mutant protein retained its nuclear localization.

Expression and Drug Resistance in K562 Cells. K562 cells were retrovirally infected by coculture with wt*MGMT* or Δ*MGMT* Am-12 producer cells. AGT activity and resistance to BG were compared in the two cell cultures without prior selection. Mean AGT levels were 25.7 fmol/μg DNA in wt*MGMT*-transduced cells, 2.7 fmol/μg DNA in Δ*MGMT*-transduced cells, and undetectable in uninfected cells. The EC_{50} for BG inactivation of AGT was <0.1 μM in wt*MGMT* compared with ≈18 μM in Δ*MGMT*-transduced cells. At 5 μM BG, ΔAGT-containing cells retained >90% activity whereas wtAGT was undetectable (Fig. 1*B*). To determine whether ΔAGT expression could increase tolerance to BG and BCNU, transduced K562 cells were exposed to 10 μM BG and/or various concentrations of BCNU and plated in methylcellulose. Clonogenic Δ*MGMT* and wt*MGMT* cells were resistant to BCNU alone. In contrast, Δ*MGMT*-transduced cells were significantly more resistant to BG and BCNU than wt*MGMT*-transduced or untransduced cells (Fig. 3). After BG treatment, the BCNU IC_{50} was 11.3 vs. 4 vs. 1.3 μM and the IC_{90} was >30 vs. 16 vs. 5 μM, for Δ*MGMT*, wt*MGMT*, and untransduced cells, respectively. Furthermore, Δ*MGMT*-transduced K562 cells maintained 20% clonogenic survival at 30 μM BCNU and BG compared with <1% of cells transduced with wt*MGMT*. Proviral integration was assessed by PCR amplification of a 152-bp *MGMT* fragment and identified in 22 of 33 colonies (67%) (data not shown). AGT immunoreactive protein levels detected in pooled colonies from wt*MGMT*-infected K562 cells were 10-fold higher than in Δ*MGMT*-transduced cells (Fig. 4).

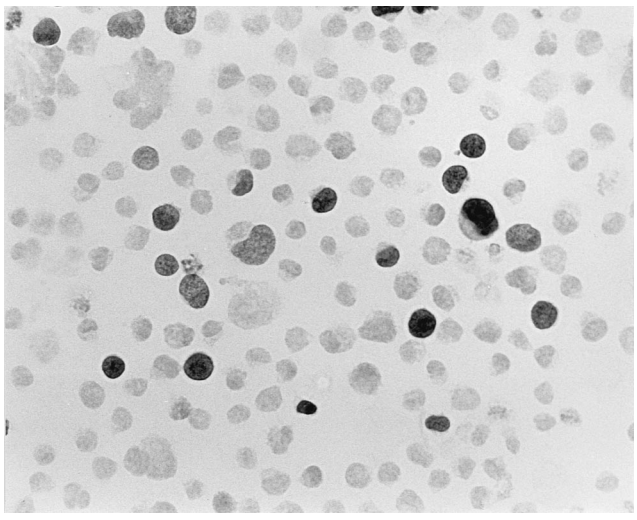

FIG. 2. Immunohistochemical detection of AGT in Δ*MGMT*-transduced K562 cells. K562 cells (1 × 105) were infected with dilutions of amphotropic supernatant collected from Am12Δ*MGMT* clone 20. At a 1:50 dilution of supernatant, ≈10% of the cells expressed nuclear human AGT when reacted with monoclonal antibody mT3.1.

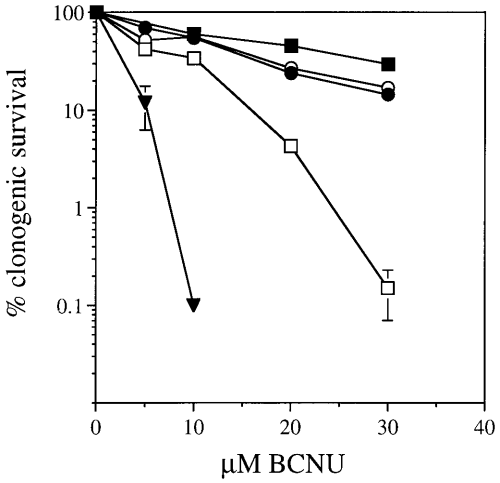

FIG. 3. Clonogenic survival of transduced K562 cells after treatment with BG and/or BCNU. K562 cells transduced with wt*MGMT* (■), Δ*MGMT* (●), and untransduced cells (▼) were treated with 0–40 μM BCNU only; wt*MGMT* (□) and Δ*MGMT* (○) were treated with 10 μM BG followed by 0–30 μM BCNU. Cells were plated in methylcellulose in triplicate, and colonies were enumerated in 7 days. Error bars represent mean ± SD.

Expression and Drug Resistance in Human CD34 Cells. The nonadherent cell count in cocultures of human CD34 cells and MFG-Δ*MGMT*, wt*MGMT*, or MFG-*lacZ* producers increased 5-fold over 96 hr due to growth factor stimulation. After infection, cells were treated with either BCNU alone or with BCNU and 10 μM BG and then plated in methylcellulose. wt*MGMT* and Δ*MGMT*-transduced CD34 cells had only a small increase in resistance to BCNU alone compared with *lacZ*-transduced cells as we have reported for wt*MGMT* (8). However, after pretreatment with 10 μM BG to deplete wtAGT, a striking resistance to BCNU was observed in Δ*MGMT*-transduced CD34 cells (Fig. 5*A*). The divergence

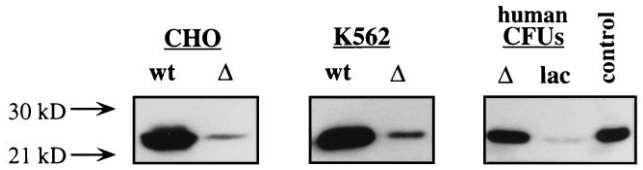

FIG. 4. Western blot analysis of human AGT expression. Protein extracts from CHO cells (lanes 1 and 2) transfected with wt*MGMT* and Δ*MGMT* and K562 cells (lanes 3 and 4) and pooled human hematopoietic progenitors (lanes 5 and 6) transduced with wt*MGMT*, Δ*MGMT*, or *lacZ* were separated on a 10% SDS/PAGE gel. Blots were immunoreacted with monoclonal antibody mT3.1, and the 22-kDa human AGT was visualized by chemiluminescent substrate. AGT activity was estimated by correlation of band intensity to a protein extract expressing known levels of AGT (lane 7).

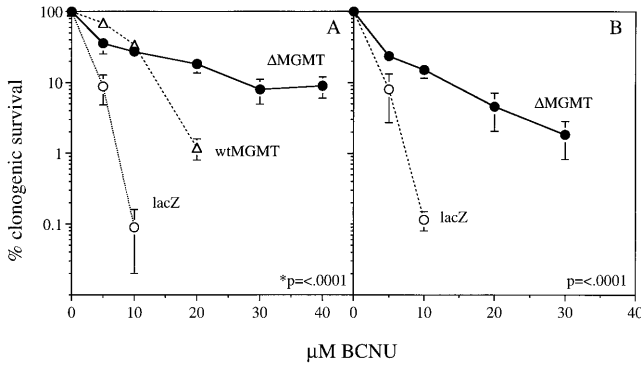

FIG. 5. Clonogenic survival of transduced CD34 cells following treatment with BG plus BCNU. CD34 cells were transduced by coculture method (*A*) or retroviral supernatant in the presence of allogenic stroma (*B*). Transduced cells were treated with 10 μM BG and 0–40 μM BCNU and plated in methylcellulose in triplicate, and colonies were enumerated in 7–10 days. Data points represent the mean ± SEM of five experiments (*A*) and three experiments (*B*) from separate donors. $P < 0.001$ for the comparison Δ*MGMT* vs. *lacZ*.

between the *lacZ*-, wt*MGMT*-, and Δ*MGMT*-transduced clonogenic progenitor cell survival increased as the dose of BCNU increased. Thus, relative to the clonogenic survival of cells transduced with *lacZ*, the survival of Δ*MGMT* was 73.7 ± 10.6 at the IC_{50} and 49.0 ± 24.2 at the IC_{90}. Approximately 25% of Δ*MGMT*-transduced clones were resistant to 10 μM BG and 10 μM BCNU, a dose that killed >99% of *lacZ*-transduced hematopoietic progenitor cells. Additionally, Δ*MGMT*-transduced progenitors maintained 20% survival compared with 1% of wt*MGMT*-transduced progenitors at 20 μM BCNU.

We also transduced CD34 cells with retroviral supernatant in the presence of human bone marrow stroma, an approach currently used in clinical protocols. Similar to results obtained by coculture transduction, Δ*MGMT*-transduced cells were significantly more resistant to the drug combination than those transduced with *lacZ* (Fig. 5*B*). The BCNU IC_{50} was 3 ± 0.5 vs. 1.5 ± 0.72, and the IC_{90} was 13.3 ± 1.5 vs. 3.6 ± 1.5 for Δ*MGMT* and *lacZ*, respectively. Following Δ*MGMT* infection, PCR amplification of proviral *MGMT* was observed in 19 of 27 (70%) individual erythroid burst-forming unit (BFU-E) and granulocyte/macrophage colony-forming unit (CFU-GM) colonies. Δ*MGMT* mRNA expression was detected in pooled colony samples by reverse transcription–PCR (Fig. 6). AGT levels detected by Western blot were 6-fold higher in Δ*MGMT*-transduced progenitor colonies than *lacZ*-transduced progenitors (Fig. 4).

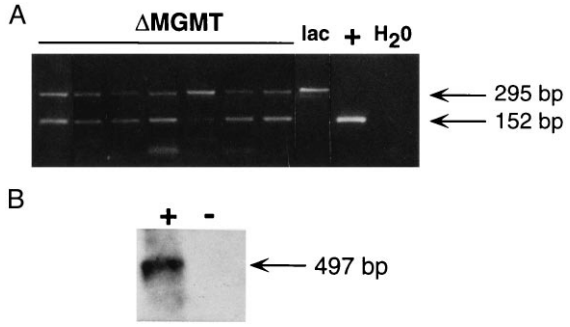

FIG. 6. Δ*MGMT* integration and expression in human hematopoietic progenitor colonies. (*A*) Representative PCR amplification of a 152-bp *MGMT* fragment and a 295-bp human dystrophin fragment as an internal control. Analysis of seven *MGMT* and one *lacZ* transduced individual progenitor colonies obtained from methylcellulose on day 10 are shown. An MFG-Δ*MGMT* plasmid was used as a positive control. (*B*) Reverse transcription–PCR amplification from pooled methylcellulose colonies as described above. After DNase treatment, a 497-bp retroviral-specific fragment was amplified in the presence of reverse transcriptase (+) but not in the absence of reverse transcriptase (−), indicating complete digestion of DNA. PCR product was detected by hybridization with a ^{32}P random primed *MGMT* cDNA.

DISCUSSION

A drug resistance gene selectively expressed in hematopoietic progenitors may provide a distinct therapeutic advantage during antineoplastic chemotherapy. In these studies, expression of the mutant drug resistance protein, G156AΔAGT, in human CD34 cells following retroviral transduction increased resistance to the combination of BG and BCNU above that seen in CD34 cells which express either endogenous or transduced wtAGT.

Enthusiasm for wt*MGMT* as a drug resistance gene emerged because cell lines lacking AGT and sensitive to BCNU became resistant after wt*MGMT* transduction (36). However, transduction of cells expressing endogenous AGT results in less enhancement of BCNU resistance than transduction of nonexpressing cells (37). In murine systems, Allay *et al.* (7) and Moritz *et al.* (38) found only about a 2-fold increase in BCNU resistance after retroviral-mediated gene transfer of wt*MGMT* into hematopoietic cells. Since transduced genes often are expressed at lower levels in human CD34 cells than in cell lines (7), it is not surprising that transduction of CD34 cells with wt*MGMT* had little effect on BCNU resistance except at high drug concentrations (8).

Δ*MGMT* gene transfer into CD34-derived colonies was efficient (70% by PCR of proviral sequences) and similar to the transduction rates reported in CD34 cells for dihydrofolate reductase using producer cell coculture and cytokine stimulation (2). Transduction of Δ*MGMT* into CD34 cells resulted in enhancement of clonogenic survival after BG and BCNU treatment. Remarkably, >30% of colony-forming progenitors appeared very resistant to BG and BCNU, compared with none of the *lacZ*-transduced cells or the nontransduced progenitors as reported (12). This degree of drug resistance compares favorably with that reported after multidrug resistance gene transfer (4, 5).

Recent evidence has shown that BG is an effective modulator of tumor–BCNU resistance in preclinical models and is well tolerated in early clinical studies (23, 27). However, it seems likely that the dose-limiting toxicity of BG and BCNU will be myelosuppression (26). Following reintroduction of Δ*MGMT*-transduced CD34 cells *in vivo*, a significant survival advantage is expected with less rather than more myelosuppression after repeated doses of BG and BCNU. Furthermore, since chloroethylnitrosoureas, procarbazine, and dacarbazine have been implicated in secondary leukemias (15, 39), it is possible that BG depletion of AGT will increase this risk, limiting the utility of the combination. Thus, another rationale for the use of Δ*MGMT* gene transfer is to protect hematopoietic cells sufficiently to prevent late oncogenic events.

These results suggest that Δ*MGMT* cDNA is a better drug-resistance gene candidate than wt*MGMT* because the relative protection of Δ*MGMT*-transduced CD34 cells against BG and BCNU is greater than observed with wt*MGMT* and BCNU alone (8, 10, 38). It is of interest that overexpression of wt*MGMT* slightly improves resistance to BG and BCNU, perhaps because of the increased rate of synthesis of AGT from the transgene. Since AGT has a much higher rate of reaction with O^6-alkylguanine DNA adducts compared with BG (40), AGT molecules synthesized after BCNU-induced DNA damage may preferentially repair the adduct rather than be inactivated by BG, which slightly increases resistance to BCNU. Nonetheless, our results indicate remarkable protection from BG and BCNU in hematopoietic cells expressing Δ*MGMT*, and it would not appear appropriate to pursue the

use of wt*MGMT* in this context. It is noteworthy that we observed higher levels of wild-type immunoreactive and functional AGT compared with mutant AGT levels in transfected CHO cells as well as transduced hematopoietic cells. This is in contrast to Loktionova and Pegg (35), who demonstrate comparable mutant and wild-type AGT levels in CHO cells. One explanation may be that the previous study compared clones selected in 80 μM BCNU, whereas our transfectants were selected based on their resistance to G418 only and not to BCNU.

Transfer of the bacterial *ada* gene, which encodes one of two bacterial alkyltransferases, into murine hematopoietic progenitors is another approach to BG-resistant alklytransferase gene transfer into hematopoietic cells. The *ada*-encoded alkyltransferase is resistant to BG when expressed in cell-free extracts and mammalian cells (41). Moore *et al.* (42) described the crystal structure of the Ada protein in which the active site cysteine is buried and must be exposed to react with the adduct at O^6 of guanine. This conformational change may be hindered by the presence of threonine at the site comparable to Gly-156 in mammalian AGTs and may explain the marked resistance to reaction with BG in both the Ada protein and ΔAGT.

Using retroviral gene transfer of the *ada* gene, Harris *et al.* (9) noted increased resistance of murine hematopoietic cells to BG and BCNU *in vitro* and a survival advantage for transplanted mice treated with the combination. Differences in survival between transduced and nontransduced cells were not as high in the Harris study as those noted here. First, there is evidence that the bacterial protein is not well-nuclear-localized and may not be an efficient DNA repair protein in eukaryotic cells even if overexpressed (37). Second, mouse AGT has a higher EC_{50} for BG than human AGT (43), so the degree of protection noted in mouse cells cannot be directly converted to that expected in human CD34 cells. Finally, it is possible that an immune response would develop against the bacterial alkyltransferase *in vivo* that would not be expected with human ΔAGT.

As a result of low AGT levels in early hematopoietic progenitors (12), these cells are susceptible to cumulative cytotoxicity from nitrosoureas and related alkylating agents. Furthermore, homozygous disruption of *MGMT* in mice results in severe myelosuppression and subsequent death within 17 days of treatment with methylnitrosourea (44). Conversely, overexpression of *MGMT* in transgenic animals provides protection against T-cell leukemias (18). Our previous studies have shown that AGT levels are not increased in CD34 cells after drug or cytokine exposure (12, 45, 46), suggesting that overexpression of Δ*MGMT* mediated by gene therapy may be the only approach to increase nitrosourea resistance in progenitor cells.

In summary, overexpression of Δ*MGMT* in CD34 cells results in resistance to the chemotherapeutic combination of BG and BCNU, suggesting that gene therapy with Δ*MGMT* rather than wt*MGMT* will provide significant, selective protection of hematopoietic cells compared with tumor cells in the clinical setting.

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