Retroviral-mediated Gene Transduction of Human Alkyltransferase Complementary DNA Confers Nitrosourea Resistance to Human Hematopoietic Progenitors

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ABSTRACT

Myelosuppression is the dose-limiting toxicity of several classes of chemotherapeutic agents (1). This toxicity limits the use of neoplastic agents and prevents dose escalation, which may potentially increase the response rate of tumors (2). The transfer and expression of drug resistance genes into hematopoietic progenitors offers the potential for alleviation of chemotherapy-induced hematopoietic injury, possibly allowing more frequent and increased drug doses. It may also reduce the incidence of late chemotherapy effects, such as marrow failure or secondary leukemias (3). Transgenic and retroviral-mediated expression of the human MDR1 gene in murine hematopoietic cells increases progenitor drug resistance in vitro. After BM transplantation, mice have reduced myelosuppression when challenged with taxol compared to mice transplanted with uninfected BM (4–10). Studies using mutant forms of the murine or human DHFR gene have shown similar findings after methotrexate treatment of transplanted mice (11–14). Recently, retroviral transduction of human CD34-enriched hematopoietic progenitors with either MDR1 (15) or DHFR (16) has been able to provide enhanced survival of human hematopoietic progenitors after cytotoxic drug treatment in vitro. In addition, drug treatment of transduced progenitors was able to increase the proportion of cells expressing increased levels of either of these drug resistance genes, suggesting that expression of drug resistance genes may be able to serve as a dominant selectable marker for genetically altered cells.

One class of agents not affected by overexpression of MDR1 or DHFR is the alkylating agents. DNA repair mediated by the O6-alkylguanine DNA AGT protein is the primary mechanism involved in resistance to nitrosoureas, triazines, and similar alkylating agents, which are cytotoxic as a consequence of forming O6-alkylguanine DNA adducts (17, 18). AGT mediates resistance to alkylating agents by an irreversible covalent transfer of an alkyl group from the O6 position of guanine to a cysteine residue within its active site (19–21). Retroviral-mediated transduction has been successful in increasing the expression of human AGT in murine hematopoietic progenitors and providing enhanced resistance to BCNU treatment. Using a myeloproliferative sarcoma virus-based retroviral vector containing the human AGT cDNA (MGMT), vMSMGMT, we achieved a 10–40-fold increase in AGT levels in multiple murine hematopoietic tissues 7–23 weeks after BM transplan-

INTRODUCTION

Myelosuppression is the dose-limiting toxicity of several classes of chemotherapeutic agents (1). This toxicity limits the use of neoplastic agents and prevents dose escalation, which may potentially increase the response rate of tumors (2). The transfer and expression of drug resistance genes into hematopoietic progenitors offers the potential for alleviation of chemotherapy-induced hematopoietic injury, possibly allowing more frequent and increased drug doses. It may also reduce the incidence of late chemotherapy effects, such as marrow failure or secondary leukemias (3). Transgenic and retroviral-mediated expression of the human MDR1 gene in murine hematopoietic cells increases progenitor drug resistance in vitro. After BM transplantation, these mice have reduced myelosuppression when challenged with taxol compared to mice transplanted with uninfected BM (4–10). Studies using mutant forms of the murine or human DHFR gene have shown similar findings after methotrexate treatment of transplanted mice (11–14). Recently, retroviral transduction of human CD34-enriched hematopoietic progenitors with either MDR1 (15) or DHFR (16) has been able to provide enhanced survival of human hematopoietic progenitors after cytotoxic drug treatment in vitro. In addition, drug treatment of transduced progenitors was able to increase the proportion of cells expressing increased levels of either of these drug resistance genes, suggesting that expression of drug resistance genes may be able to serve as a dominant selectable marker for genetically altered cells.

One class of agents not affected by overexpression of MDR1 or DHFR is the alkylating agents. DNA repair mediated by the O6-alkylguanine DNA AGT protein is the primary mechanism involved in resistance to nitrosoureas, triazines, and similar alkylating agents, which are cytotoxic as a consequence of forming O6-alkylguanine DNA adducts (17, 18). AGT mediates resistance to alkylating agents by an irreversible covalent transfer of an alkyl group from the O6 position of guanine to a cysteine residue within its active site (19–21). Retroviral-mediated transduction has been successful in increasing the expression of human AGT in murine hematopoietic progenitors and providing enhanced resistance to BCNU treatment. Using a myeloproliferative sarcoma virus-based retroviral vector containing the human AGT cDNA (MGMT), vMSMGMT, we achieved a 10–40-fold increase in AGT levels in multiple murine hematopoietic tissues 7–23 weeks after BM transplan-

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4 The abbreviations used are: MDR1, multidrug resistance; MGMT, methylguanine methyltransferase; BCNU, 1,3-bis-(2-chloroethyl) nitrosourea; IL, interleukin; AGT, alkyltransferase; BM, bone marrow; DHFR, dihydrofolate reductase. CFU, colony-forming unit; O6bG, O6-benzylguanine; O6MeG, O6-methylguanine.
tation with vM5MGMT-transduced BM (22). This increased DNA repair capacity provided hematopoietic progenitors derived from these mice with a significant increase in nitrosourea resistance. In a separate study, Moriz et al. (23) designed a model to mimic the delayed myelotoxicity associated with nitrosourea therapy, using weekly BCNU administration combined with biweekly infusion of marrow progenitors. Animals receiving mock-transduced cells suffered from severe pancytopenia, whereas animals receiving MGMT-transduced BM progenitors had significantly higher hematocrits and leukocyte and platelet counts. In addition, recently Maze et al. (24) have shown increased myeloprotection after BCNU treatment of mice transplanted with MGMT-transduced immature hematopoietic progenitors.

In this report, we use vM5MGMT to transduce CD34+ enriched human hematopoietic progenitors. We show increased AGT levels leading to increased survival of progenitors after exposure to BCNU in vitro. In addition, initial experiments suggest that BCNU can be used as a selective agent to enrich for AGT-expressing cells.

MATERIALS AND METHODS

Viral Vectors and Producer Cells. vM5MGMT and vM5LacZ, both derived from the myeloproliferative sarcoma virus (kindly provided by Dr. W. Ostertag, University of Hamburg, Germany), have been described previously (22). Producer cell lines were generated by transfection of pM5MGMT or pM5LacZ into GP + E86 and GP + envAM12 cell lines (kindly provided by Dr. A. Bank, Columbia University, New York, NY) and using a modified supernatant “ping-pong” to increase viral titer (22, 25). Titer of the AM12-derived vM5MGMT- and vM5LacZ-producing cell line were 1 × 10^5 neor CFU/ml as measured by limiting dilution on NIH 3T3 cells. Virus preparations were found to be free of replication competent retrovirus by previously described methods (22).

Human BM Harvest and CD34+ Separation. BM aspirates were obtained from the iliac crest of adults undergoing marrow harvest as normal donors or in preparation for autologous BM transplantation. All donors gave informed consent and all marrow samples were histologically normal.

Red blood cells were lysed from whole marrow using 60 mM ammonium chloride, 10 mM potassium bicarbonate, and 0.01 mM tetrasodium EDTA at 37°C. The cells were washed several times with 1% BSA in PBS and used as the starting cell population for CD34+ separation. CD34+ cells were positively selected by immunoabsorption column CEPRATE (S. Heimfeld, CellPro, Inc., Bothell, WA) according to the manufacturer's instructions.

Flow cytometry for CD34 content using the HPCA (human progenitor cell antibody) human anti-CD34 monoclonal antibody and a KLH isotype monoclonal antibody (both from Becton Dickinson, San Jose, CA) and clonogenic progenitor assays (see below) were performed before and after column separation.

CD34-enriched Cell Transduction. CD34-enriched cells were resuspended at 1 × 10^4 to 5 × 10^4 cells/ml in X-vivo-10 (BioWhittaker, Wakersville, MD) supplemented with 15% heat inactivated FCS (Hyclone Laboratories, Logan, UT), 100 ng/ml stem cell factor (kindly provided by Dr. N. K. Alton, Amgen, Thousand Oaks, CA), 100 units/ml IL-3, 50 units/ml IL-6 (both kindly provided by Genetics Institute, Cambridge, MA), and 6 µg/ml polybrene (Sigma Chemical Co., St. Louis, MO), and overlaid on mitomycin C-treated AM12-vM5MGMT or AM12-vM5LacZ producer cells. Forty-eight h later, 1/3 volume was removed, and the cells were pelleted by centrifugation and resuspended in fresh complete medium containing cytokines and polybrene at the above concentrations and returned to the coculture. Ninety-six h after the beginning of the coculture, nonadherent hematopoietic cells were collected and analyzed for gene transduction, AGT expression, and BCNU resistance.

In Vitro BCNU Treatment. vM5MGMT- or vM5LacZ-transduced CD34+ cells (5 × 10^5) were resuspended in serum-free X-vivo-10 and incubated with various concentrations of BCNU for 2 h at 37°C as described previously (22). Cells were washed in serum-free X-vivo-10 and either returned to cytokine-supplemented culture (using the same combination and concentrations of cytokines as above) or used for progenitor assays (see below).

Progenitor Assay. vM5MGMT- or vM5LacZ-transduced CD34+ cells (3 × 10^5) were mixed with methylcellulose (Stem Cell Technologies, Vancouver, British Columbia, Canada), 100 ng/ml stem cell factor, 100 units/ml IL-3, 2 units/ml erythropoietin (Amgen, Thousand Oaks, CA), 100 units/ml GM-CSF (kindly provided by Sandoz Research Institute, Nutley, NJ) and 0.1 mM hemin (Sigma, St. Louis, MO), and plated in triplicate at 37°C and 5% CO2. After 12 days, colonies greater than 50 cells were enumerated. Differential survival between treatment groups was analyzed by paired t tests of the mean of the BCNU IC50 and comparisons at each dose of BCNU. Large colonies were isolated for PCR analysis for proviral integration. Some plates were rinsed with PBS, and the progenitor colonies were pooled for Western blot analysis.

PCR Provirus Analysis of Progenitor Colonies. Individual progenitor colonies were resuspended in 30 µl of water and boiled for 8 min. One µl of a 10 mg/ml proteinase K solution was added to each sample, the colonies were incubated for 2 h at 55°C and boiled for 8 min, and 10 µl were used for PCR. A 152-bp human MGMT cDNA-specific fragment was amplified (using previously described primers; Ref. 22) with a 295-bp human dystrophin gene fragment using the proximal primer 5’ TCACCTGCTTGTGCCCCAGG 3’ and the distal primer 5’ GAAAATGTATATCGAGCACGATAAA 3’. The fragments were separated on an agarose gel and visualized by either ethidium bromide stain or Southern blot.

AGT Assays. AGT activity was measured as previously described (26). Briefly, enzyme activity was measured as 3H methyl groups removed from [3H]O6-MeG present in calf thymus DNA alkylated with [3H]methylirnitosourea (specific activity of 0.047 fmol O6-MeG/µg DNA). The alkylated [3H]O6-MeG and N-7-methylguanine bases were separated by high-performance liquid chromatography and quantified by liquid scintillation. N-7-methylguanine was used as the internal standard. AGT activity was expressed as fmol O6-MeG removed/mg protein.

Western Blot Analysis. CD34 cells and pooled progenitor colonies were lysed by sonication and boiling, and 50 µg of protein extract were separated by SDS-PAGE. Detection using the anti-human AGT monoclonal antibody mT3.1 (kindly pro-
Table 1  Characterization of the CD34-enriched cell population after separation with CellPro CEPRATE immunoaffinity columns.
Red-cell lysed human bone marrow cells were incubated with a biotin-conjugated anti-CD34 monoclonal antibody and separated using CEPRATE columns containing avidin coated beads (CellPro). Flow cytometry for CD34 content and clonogenic progenitor assays were performed before and after separation.

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<th>Experiment</th>
<th>Fold enrichment</th>
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* ND, not determined.

RESULTS

CD34 Separation and Characterization. Table 1 shows that progenitors measured by in vitro clonogenic assays were enriched 66-±23-fold after column separation (range, 38-100-fold; n = 5) with an average progenitor yield of 58 ± 11.5% (range, 43-71%; n = 5). Analysis of cells from two experiments using flow cytometry showed a 100-fold enrichment of CD34+ cells, with 82% recovery and a purity of 54%.

Retroviral Transduction of CD34-enriched Cells. The CD34+ -enriched population of cells was retrovirally transduced by coculture with amphotropic vM5MGMT or vMSLacZ producer cells over 96 h with hematopoietic cytokine stimulation. The nucleated cell number increased an average of 5.3-±4.3-fold (range, 2-14.6-fold; n = 7) during the coculture. Immediately after coculture, nonadherent cells were collected and plated in methylcellulose, and individual progenitor colonies were analyzed by PCR for evidence of MGMT proviral integration. Table 2 shows that 38 of 54 colonies examined from five experiments had evidence of proviral integration, indicating a transduction efficiency of 70%. Cultures of vM5MGMT- and vM5LacZ-transduced progenitors without prior BCNU exposure contained similar numbers and sizes of colonies, indicating that vM5MGMT transduction and expression did not confer a growth advantage in clonogenic progenitors. Fig. 1 shows representative PCR amplifications.

Expression of Human MGMT and Nitrosourea Resistance in vM5MGMT-transduced CD34+ Cells. AGT expression of human MGMT was evaluated in vM5MGMT- and vM5LacZ-transduced CD34+ -derived progenitor colonies before nitrosourea exposure by Western blot and biochemical assay. The AGT expression of pooled progenitor colonies derived from vM5LacZ- and vM5MGMT-transduced progenitors from three separate experiments was not significantly different (123 ± 19 and 163 ± 104 fmol/mg protein, respectively; P = 0.6).

To determine whether MGMT-transduced progenitors have increased survival after nitrosourea treatment, vM5MGMT- and vM5LacZ-transduced progenitors were assayed for resistance to BCNU. Fig. 2 shows survival curves from a representative experiment in which vM5MGMT-transduced progenitors showed significantly increased survival at 30-50 μM BCNU, compared to vM5LacZ-transduced progenitors (P < 0.01). In two separate experiments, vM5MGMT-transduced progenitors were over 2-fold more resistant to 30-50 μM BCNU than concurrently vM5LacZ-transduced and BCNU treated progenitors (P < 0.003).

The potential for using BCNU as a selective agent for vM5MGMT-transduced hematopoietic progenitors was further investigated. vM5MGMT- and vMSLacZ-transduced CD34+ cells were treated with 20 μM BCNU immediately after infection and returned to liquid culture with addition of hematopoietic cytokines for 48 h. Cells were then cultured in methylcellulose for colony formation, and at this point, AGT levels in MGMT-transduced progenitors increased an average of 2-fold to 330 ± 287 fmol/mg protein with no AGT increase in concurrently treated LacZ-transduced progenitors, which is shown by Western blot in Fig. 3. Likewise, survival of clonogenic cells from cultures of vM5MGMT-transduced progenitors that have been exposed to BCNU and cultured for 48 h before progenitor assay was 4.7% compared to 0.05% of vM5LacZ-transduced progenitors. The lower overall survival of clonogenic cells compared to that seen above (Fig. 2) suggests that the effective BCNU concentration may have been higher or that liquid culture expansion of cells before plating in methylcellulose diluted the concentration of clonal progenitors. These data suggest that a small fraction of BCNU-resistant progenitors exists within the vM5MGMT-transduced CD34+ cell population, which may be enriched by in vitro BCNU treatment.

DISCUSSION

We report conditions for high-efficiency retroviral-mediated transduction of the human MGMT cDNA into BM-derived, CD34-enriched progenitors. 70% of the individual progenitor colonies assayed by PCR immediately after infection showed evidence of MGMT gene transfer. We suggest that the increase in AGT levels obtained after transduction was sufficiently high in at least a proportion of MGMT-transduced progenitors to...
result in a significant increase in BCNU resistance in comparison to concurrently analyzed LacZ-transduced progenitors. The heterogeneity of MGMT transgene expression in the transduced, unselected cell population may explain our inability to detect increased AGT levels by Western blot and biochemical assay. In addition, in vitro BCNU treatment followed by cytokine-supplemented liquid culture led to higher survival of MGMT-transduced progenitors compared to progenitors transduced with LacZ.

The data presented here used a 96-h coculture of CD34-enriched BM cells and AM12 amphotropic MGMT producer cells supplemented with hematopoietic growth factors. This was a more effective means of transduction of CD34-derived progenitors, in our experience, than the more commonly used protocol using a 48-h prestimulation of hematopoietic cells.

Fig. 1 vMSMGMT integration in CD34-derived progenitors. CD34-enriched BM cells were transduced with vMSMGMT by a 96-h coculture and cytokine stimulation. Cells were treated with BCNU and plated in a hematopoietic progenitor assay. Individual colonies were isolated and used in a PCR amplification with primers specific for a 152-bp fragment of the human MGMT cDNA and primers specific for a 290-bp fragment of the human dystrophin gene as an internal control. Shown are PCR amplifications of erythroid (BFU-E) and myeloid (CFU-GM) progenitor colonies treated with 0 µM or 10 µM BCNU before plating. Control is a vMSLacZ-transduced progenitor colony. Positive (Pos) is a BM-derived progenitor from a mouse transgenic for the human MGMT cDNA.

Fig. 2 BCNU resistance of vMSMGMT-transduced CD34-derived progenitors. CD34-enriched BM cells were transduced with vMSMGMT or vMSLacZ by a 96-h coculture and cytokine stimulation. Cells were then treated with BCNU, plated in a hematopoietic progenitor assay, and scored for survival relative to an untreated control (P < 0.01 for 30, 40, and 50 µM BCNU).

Fig. 3 AGT expression in vMSMGMT- and vMSLacZ-transduced CD34-enriched BM cells. Protein extracts (50 µg) of vMSMGMT- or vMSLacZ-transduced CD34+ cells, immediately after infection (before BCNU treatment) or after 20 µM BCNU treatment and cytokine-supplemented culture, were separated by SDS-PAGE. The subsequent blot was probed with a monoclonal antibody (mT3.1; Ref. 27) directed against the human AGT protein and developed using chemiluminescence.
before coculture with producer cells (15, 28, 29). Our rationale for pursuing this experimental approach was the observation that during mock infections, the majority of progenitor expansion occurred during the first 48 h of cytokine-stimulated ex vivo culture (30). Because proviral integration requires cellular division (31, 32), this suggests that proliferation of CD34-enriched progenitors assayed in semisolid medium (CFU), or perhaps a pre-CFU cell, may be maximal in the period immediately after introduction into cytokine-supplemented culture. Thus, exposure to replication-defective retrovirus at this time may be optimal to obtain high levels of transduction of lineage-restricted marrow-derived hematopoietic progenitors.

Increased MGMT expression by committed progenitors may have clinical importance for alleviation of nitrosourea-induced myelotoxicity even if transduction of pluripotent repopulating stem cells is insufficient. Moritz et al. (23) showed that mice that had received repeated infusions of MGMT-transduced hematopoietic progenitors had significantly increased peripheral blood cell counts (white blood cells, hematocrit, and platelets) during multiple rounds of BCNU administration in comparison to mice receiving infusions of unmodified marrow cells (23). Thus, increased AGT expression in committed progenitors may result in less myelosuppression, especially after repeated drug exposure.

In addition, MGMT, like other drug resistance genes (9, 10), can potentially serve as a dominant selectable marker for enrichment of genetically altered cells. In vitro BCNU treatment followed by culture in cytokine-supplemented medium of MGMT-transduced CD34+ cells resulted in cells with increased AGT expression and increased BCNU resistance, whereas no such effect was observed with LacZ-transduced control cells.

Furthermore, there is no evidence that AGT can be induced in human CD34+ cells or in mouse or rat BM or hematopoietic cell lines after exposure to nitrosoureas, cytokines, or differentiating agents (33–35). We have also shown that repeated administration of BCNU to mice transplanted with MGMT-transduced marrow cells significantly increased the resistance of hematopoietic progenitors to in vitro BCNU treatment, increased the proportion of these progenitors with evidence of retroviral integration, and increased the human AGT levels of mouse BM relative to mice that were transplanted with MGMT-transduced cells but not treated with BCNU (36). These data suggest that BCNU treatment will be able to enrich for cells that have been transduced with MGMT and a second, nonselectable gene.

One application using MGMT as a dominant selectable marker could use retroviral vectors that rely on splicing, such as eDNA or an internal promoter for expression of the selectable marker. Alternatively, efficient expression of two (or more) genes has recently been shown to occur by exploiting virus-derived ribosomal entry sites, termed internal ribosome entry sites (37–39). An internal ribosome entry site can be placed between two genes that are transcribed from the same promoter, creating a bicistronic transcript within the retroviral vector. Notably, one group has used this strategy with MDR1 serving as the selectable marker gene, and genes such as human glucose-6-phosphatase (40), herpes simplex virus thymidine kinase (41), or human α-galactosidase A (42) as part of the bicistronic message. However, MGMT may be a more appropriate gene for use as a selectable marker in hematopoietic stem and progenitor cells than MDR1, due to the small size of the human MGMT cDNA (700 bp versus approximately 4000 bp for the MDR1 cDNA) and the relatively low expression of MGMT in myeloid precursors (25, 43) in comparison to the relatively high expression of MDR1 in immature hematopoietic progenitors (44, 45).

Our data indicate a modest but significant survival advantage of MGMT-transduced progenitors relative to those transduced with the LacZ gene. This may be the result of increased AGT expression within a minor population of MGMT-transduced, unselected cells. In these studies, the rate-limiting factor appears to be the level of AGT expression from the transgene, which was low, and not the rate of transduction, which was high. To improve the potential therapeutic efficacy of this approach, a gene transfer technique that markedly increases AGT expression is needed, as has been possible in murine but not human hematopoietic cells. An alternative strategy to increase the survival advantage of MGMT-transduced progenitors would be to use the AGT inhibitor O6-BG (46–48), with an AGT molecule that is resistant to O6-BG, such as the bacterial (49) or mouse (50) homologues or the more recently characterized mutants of the human protein, although these have yet to be shown to function in mammalian cells (51, 52). The combination of BCNU and O6-BG treatment of cells expressing one of the O6-BG-resistant proteins should enhance the selective advantage compared to BCNU treatment alone. Recently, Harris et al. (53) have shown that retroviral-mediated exogenous expression of the bacterial AGT homologue, ada, protects mouse BM cells from O6-BG and BCNU treatment. However, for these proteins to function efficiently in providing resistance to nitrosoureas, evidence of protein stability, enzymatic activity, and nuclear localization in human hematopoietic cells need to be established.

These studies establish the feasibility of decreasing nitrosourea-induced myelosuppression using retroviral-mediated transfer and expression of the human MGMT cDNA in human CD34+ cells. Experiments designed to address coexpression of MGMT with a second nonselectable gene in hematopoietic progenitors will further define the ability of AGT expression to serve in a dominant selectable marker strategy.

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Retroviral-mediated gene transduction of human alkyltransferase complementary DNA confers nitrosourea resistance to human hematopoietic progenitors.


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