In Vivo Effects of Myeloablative Alkylator Therapy on Survival and Differentiation of MGMT$^{P140K}$-Transduced Human G-CSF-Mobilized Peripheral Blood Cells

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High-intensity alkylator-based chemotherapy is required to eradicate tumors expressing high levels of O6-methylguanine DNA methyltransferase (MGMT). This treatment, however, can lead to life-threatening myelosuppression. We investigated a gene therapy strategy to protect human granulocyte colony-stimulating factor-mobilized peripheral blood CD34+ cells (MPB) from a high-intensity alkylator-based regimen. We transduced MPB with an oncoretroviral vector that coexpresses MGMT$^{P140K}$ and the enhanced green fluorescent protein (EGFP) ($n = 5$ donors). At 4 weeks posttransplantation into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice, cohorts were not treated or were treated with low- or high-intensity alkylating chemotherapy. In the high-intensity-treated cohort, it was necessary to infuse NOD/SCID bone marrow (BM) to alleviate hematopoietic toxicity. At 8 weeks posttreatment, human CD45+ cells in the BM of mice treated with either regimen were EGFP+ and contained MGMT-specific DNA repair activity. In cohorts receiving low-intensity therapy, both primitive and mature hematopoietic cells were present in the BM. Although B-lymphoid and myeloid cells were resistant to in vivo drug treatment in cohorts that received high-intensity therapy, no human CD34+ cells or B-cell precursors were detected. These data suggest that improved strategies to optimize repair of DNA damage in primitive human hematopoietic cells are needed when using high-intensity anti-cancer therapy.

Key Words: gene therapy, hematopoietic stem cell, NOD/SCID mice, SCID-repopulating cell, O6-methylguanine DNA methyltransferase, O6-benzylguanine, BCNU, G-CSF-mobilized peripheral blood

INTRODUCTION

Maintenance of genome stability in hematopoietic stem and progenitor cells (HSC) is essential for normal blood cell development. Survival of HSC and their progeny can be severely compromised during exposure to DNA-damaging drugs used in anti-cancer therapy due to low levels of endogenous DNA repair activity [1–3]. In terms of anti-cancer therapy, generation of HSC that efficiently repair DNA damage due to chemotherapy may protect patients from life-threatening cytopenias commonly observed following dose-intensified therapy. In a recent phase II clinical trial, patients with nitrosourea-resistant gliomas were simultaneously treated with O6-benzylguanine (6BG) to deplete the DNA repair protein O6-methylguanine DNA methyltransferase (MGMT) and the DNA damaging agent 1, 3-bis (2-chloroethyl)-1-nitrosurea (BCNU) [4]. Although lack of tumor progression was transiently observed in some patients, effective dose-escalation therapy could not be achieved due to severe hematopoietic toxicity. These studies provide clinical proof that strategies protecting HSC during dose-intensified therapy are indeed clearly needed in relapsed patients requiring high-dose alkylator therapy.

Numerous transplant studies have convincingly proven that murine stem cells could be selected in vivo with 6BG/BCNU, 6BG/temozolomide, or 6BG/CCNU [5–21]. In addition, studies performed by Neff et al. [11,22] used dose-escalation regimens in a canine transplant model...
and demonstrated selection of MGMT<sup>P140K</sup>-expressing cells over time with no signs of hematotoxicity or overt multiorgan toxicity reported. We previously used the severe combined immunodeficient (SCID)-repopulating cell (SRC) assay to investigate the extent to which human SRCs and their progeny were selected in vivo from submyeloablative doses of 6BG and BCNU [7]. Human HSC derived from umbilical cord blood (UCB) or granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood (MPB) that expressed MGMT<sup>P140K</sup> could be selected in vivo by nonmyeloablative doses of 6BG and BCNU. Gerson and colleagues also reported similar results using MGMT<sup>P140K</sup>-transduced UCB in the nonobese diabetic (NOD)/SCID xenograft model [9].

Treatment of nitrosourea-resistant cancers will likely require high doses of chemotherapy in the myeloablative range necessitating a stem-cell transplant. In addition, efficient and prolonged depletion of wild-type MGMT (wtMGMT) by agents such as 6BG will be necessary so that tumor DNA damage as a consequence of treatment with alkylating agents can be optimally accomplished [4,23,24]. We and others previously demonstrated simultaneous protection of murine stem cells with mutant MGMT protein and significant lack of disease progression of nitrosourea-resistant tumors in NOD/SCID mice treated with 6BG/BCNU [13,25]. An aggressive myeloablative 6BG/BCNU regimen was used in our study. Kreklau et al. [26,27] demonstrated that wtMGMT begins to regenerate within hours after delivery of 6BG; therefore, one dose may not allow time for sufficient DNA crosslinks to be generated prior to de novo synthesis of wtMGMT. They also found that administration of two boluses of 6BG 8 h apart led to a prolonged depletion of wtMGMT in a human glioma (SF767) that expresses high levels of wtMGMT [26,27]. Our group tested a myeloablative dosing schema that consisted of two boluses of 6BG delivered 8 h apart combined with one dose of BCNU delivered 1 h after the first bolus of 6BG. NOD/SCID mice were engrafted with a human glioma that expresses high levels of wtMGMT (SF767). Following transplant with murine bone marrow (BM) expressing MGMT<sup>P140K</sup>, the mice received two cycles of the 6BG double-bolus regimen over a 2-week period. A significant reduction in the growth of the engrafted glioma was observed [13].

The extent to which human HSC can be protected in vivo by MGMT<sup>P140K</sup> during delivery of high doses of alkylator therapy that kills cancer cells has not been investigated. In this report, we compare the outcome of administering a low-dose 6BG/BCNU regimen versus a high-dose regimen to NOD/SCID mice transplanted with MGMT<sup>P140K</sup>-transduced MPB. The in vivo model used here provides the most stringent test of human HSC protection and self-renewal capacity tested to date in vivo. We found that low numbers of human MPB cells were protected following delivery of the myeloablative regimen and that these cells were limited to mature lymphoid and myeloid cells. This model system can now be used to optimize protection of human HSC during high-intensity alkylator therapy.

**RESULTS AND DISCUSSION**

Administration of high-dose alkylator-based therapy that is myeloablative at initial diagnosis or immediately following cancer relapse may be the most effective means of eradicating drug-resistant tumors that express high levels of wtMGMT. In the current study we used the NOD/SCID xenograft model to evaluate the impact of high-dose alkylator therapy on human hematopoiesis. We used MPB CD34<sup>+</sup> cells since this is the HSC source utilized in human gene therapy trials focused on preventing myelosuppression in cancer patients undergoing high-dose therapy [28,29].

Transduction Efficiency of Human CD34<sup>+</sup> Cells Prior to Transplantation

We stimulated human CD34<sup>+</sup> cells isolated from MPB for 48 h with a cytokine cocktail consisting of G-CSF, stem cell factor (SCF), and thrombopoietin (TPO) and transduced them with a gibbon ape leukemia virus (GALV)- pseudotyped oncoretroviral vector that coexpresses MGMT<sup>P140K</sup> and enhanced green fluorescent protein (EGFP) (SF1-P140K). We performed five independent transplant experiments (MPB 1–5) using CD34<sup>+</sup> MPB cells isolated from five normal donors. EGFP expression was used to determine the transduction efficiency of the MPB prior to transplantation. The transduction efficiency was 35.8 ± 11% (mean ± standard deviation) for CD34<sup>+</sup> cells and 45.2 ± 8% for committed progenitor cells (for details see supplemental information online).

Development of a Murine BM support Model to Evaluate the Impact of High-Dose 6BG/BCNU Chemotherapy on Human Hematopoiesis

Our primary objective was to evaluate to what extent human MPB CD34<sup>+</sup> cells transduced with the SF1-P140K vector were protected from a low-intensity regimen consisting of 6BG and BCNU that is in the submyeloablative range (Fig. 1A, see low dose) versus a high-intensity regimen that is myeloablative (Fig. 1A, see high dose). The low-dose regimen served as an excellent control with which to compare the impact of the high-dose regimen on human hematopoiesis since we had shown previously that this regimen led to selection of human clonogenic cells in the NOD/SCID xenograft model [7]. In pilot studies we initially transplanted NOD/SCID mice with SF1-P140K-transduced human CD34<sup>+</sup> cells and delivered two cycles of either low-dose or high-dose therapy commencing at 4 weeks posttransplant. Transplanted mice receiving the high-dose therapy died of severe pancytopenia within 7–10 days posttreatment, although transplanted mice treated with low-dose
chemotherapy survived (data not shown). These data suggested that although human hematopoietic cells could repopulate the BM, they did not contribute to the survival of the animal under such genotoxic conditions as delivery of high-dose chemotherapy. Therefore, to prevent life-threatening myelosuppression, we infused in vivo selected SF1-P140K-NOD/SCID BM into transplanted mice undergoing high-dose chemotherapy (Fig. 1, high dose). We found that by maintaining functional murine hematopoiesis, 85–100% of the mice survived (data not shown). This approach was used in MPB 5 and gave results similar to those observed in MPB 1–4.

Assessment of Transduced Hematopoietic Cells Following Delivery of Low- or High-Dose Chemotherapy

At 8 weeks postchemotherapy, we analyzed the NOD/SCID BM for the presence of human cells. In contrast to nontreated mice, the vast majority of the human CD45+ cells expressed EGFP in mice treated with low- or high-dose chemotherapy (Fig. 1B). Representative examples from three of the five independent experiments are shown. As expected in mice receiving high-dose therapy, human and mouse cells that expressed EGFP were present (Fig. 1B, Hi-MPB 3 and Hi-MPB 4). Flowcytometric analyses indicated that the EGFP+ cells that did not express human CD45 expressed the murine EGFP.
CD45 antigen (data not shown). Since nontransduced NOD/SCID BM was used to maintain murine hematopoiesis in MPB5, no murine EGFP⁺ cells were present (Fig. 1B, Hi-MPB 5).

To evaluate the effects of low- versus high-dose therapy on human hematopoiesis, we determined the percentage of human cell engraftment (Fig. 2A, %huCD45⁺) and the percentage of transduced human cells (Fig. 2B, %huCD45⁺EGFP⁺) using samples of BM from transplanted mice. The majority of the mice survived the low- and high-dose regimens (Fig. 2). In MPB 2 and MPB 4, two mice in cohorts receiving the high-dose regimen died 5–6 days after the second cycle of chemotherapy. Analysis of BM cellularity indicated that the marrow was hypocellular. In MPB 3, one mouse in the nontreated group died at 6 weeks posttransplant and in MPB 5 one mouse in the cohort receiving the low-dose regimen died after the second cycle of chemotherapy. Consistent with previous transplant studies using MPB, human cell chimerism varied among the five MPB donors, with the drug-treated cohorts exhibiting lower levels of human cell chimerism (Fig. 2A) [7,30]. Under both low- and high-dose regimens, the majority of the remaining human cells expressed EGFP (Fig. 2B). The statistical analyses of these data are presented in Table 1. The percentage of huCD45⁺ cells in the BM was marginally decreased in the high-dose cohort compared to the low-dose cohort (P = 0.095), while the percentage of huCD45⁺ cells was significantly decreased in both the low- and the high-dose cohorts compared to the NT group (both P < 0.0001). The percentage of huC-D45⁺EGFP⁺ cells was significantly increased in all comparisons (Hi vs Low, Low vs NT, and Hi vs NT) (Table 1). The increasing numerical order for the percentage of human cells that were EGFP⁺ was NT < Low < Hi. These data indicate that although human engraftment levels decreased, the remaining cells contained the SF1-P140K vector and were resistant to in vivo drug treatment. In addition, we found no human cells in the spleens of drug-treated mice when engraftment was ≤5%, which was the

FIG. 2. Effect of low- or high-dose 6BG/BCNU on human cell engraftment and transduction efficiency. At 16 weeks posttransplantation, the BM of NOD/SCID mice was analyzed for (A) the percentage of human cell engraftment (%huCD45⁺) and (B) the percentage of human cells that are EGFP⁺ (%huCD45⁺EGFP⁺). Five independent experiments were performed (MPB 1–5). Data are presented as the means (diamonds) ± the standard error (lines). See Table 1 for statistical significance. NT, nontreated; Lo, low-dose chemotherapy; Hi, high-dose chemotherapy; n, the number of mice that survived and were analyzed (numerator) versus the number of mice initially transplanted (denominator).
We determined the impact of the low- or high-dose chemotherapy following delivery of low- or high-dose regimen (Table 1) suggests that the remaining CD33+ cells must be somewhat immature since they could survive for 8 weeks postchemotherapy but were still fairly differentiated overall since they had progressed low-dose chemotherapy but not high-dose chemotherapy (Fig. 4). In sharp contrast, even though we collected and analyzed via flow cytometry large numbers of events from the BM of transplanted mice that received high-dose chemotherapy, EGFP+CD34+CD38− and EGFP+CD34+CD38+ cells were not detected (ND) (Fig. 4). The BM of the transplanted mice (NT, low, and high) was also analyzed for clonogenic activity by the colony-forming unit (CFU) assay (Table 2). We analyzed large numbers of progenitor plates since the transduction level of the human cells in the nontreated mice was higher than the engraftment level of the human cells in the drug-treated mice were low. The number of EGFP+ CFU detected in the nontreated and treated animals was consistent with our previously published study [7]. The presence of hematopoietic cells that still possessed clonogenic activity in transplanted mice receiving the high-dose regimen was extremely limited. We classified a colony as clonogenic if it contained 50 cells or greater since this is the accepted cutoff in the CFU assay. In the BM of these mice, we did occasionally observe nonclonogenic cell clusters in the methylcellulose that contained ~10–30 cells per cluster (data not shown). The two progenitor colonies that were detected in the BM of transplanted mice receiving the high-dose regimen were of myeloid origin (i.e., CFU-GM). These data suggest that although MGMTP140K expression levels were sufficient to protect committed progenitors under low-dose therapy, expression levels were not adequate to maintain a pool of detectable committed progenitors following high-dose therapy.

We next determined the frequency of EGFP+CD34+CD19− (B-lymphoid progenitors) and EGFP+CD34−CD19+ (mature B-lymphoid cells) (Fig. 5A). Under low-dose therapy, both B-lymphoid progenitors and mature B-lymphoid cells were present and the majority of the cells expressed EGFP. In contrast, in the transplanted mice that received high-dose therapy, mature B-lymphoid cells but not B-lymphoid progenitors were present, again indicating that the more primitive hematopoietic cells were not protected adequately during high-dose chemotherapy. Similar to our findings showing that lymphopoiesis of transduced human cells was diminished following chemotherapy in the high-dose range, the appearance of transduced lymphocytes in a dog transplanted with allogeneic MGMTP140K- transduced BM appeared to be compromised if dosing was increased above a certain threshold level [11].

Human myeloid cells (huCD45+CD33+) were protected in the BM of transplanted mice receiving either low- or high-dose therapy (Fig. 5B) and the vast majority of these cells expressed EGFP. The virtual lack of CFU-GM progenitors in transplanted mice that received the high-dose regimen (Table 2) suggests that the remaining CD33+ cells must be somewhat immature since they could survive for 8 weeks postchemotherapy but were still fairly differentiated overall since they had progressed

### Analysis of Multilineage Engraftment of Human Cells Following Delivery of Low- or High-Dose Chemotherapy

We determined the impact of the low- or high-dose therapy on the maintenance of primitive human CD34+ cells and differentiation of lymphomyeloid cell lineages by flow cytometry. An enrichment of EGFP+CD34+CD38− and EGFP+CD34+CD38+ cells was evident in mice receiv

<table>
<thead>
<tr>
<th>Variable</th>
<th>Comparison</th>
<th>Estimate</th>
<th>P value</th>
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<tbody>
<tr>
<td>% huCD45</td>
<td>Hi vs Low</td>
<td>−0.052</td>
<td>0.095</td>
</tr>
<tr>
<td></td>
<td>Low vs NT</td>
<td>−0.187</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Hi vs NT</td>
<td>−0.239</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>% huCD45+EGFP</td>
<td>Hi vs Low</td>
<td>0.143</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Low vs NT</td>
<td>0.668</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Hi vs NT</td>
<td>0.811</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>% huCD19</td>
<td>Hi vs Low</td>
<td>−0.191</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>Low vs NT</td>
<td>0.130</td>
<td>0.120</td>
</tr>
<tr>
<td></td>
<td>Hi vs NT</td>
<td>−0.061</td>
<td>0.459</td>
</tr>
<tr>
<td>% huCD33</td>
<td>Hi vs Low</td>
<td>−0.009</td>
<td>0.767</td>
</tr>
<tr>
<td></td>
<td>Low vs NT</td>
<td>0.747</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>Hi vs NT</td>
<td>0.738</td>
<td>0.016</td>
</tr>
<tr>
<td>% huCD34</td>
<td>Low vs NT</td>
<td>−0.551</td>
<td>0.130</td>
</tr>
<tr>
<td></td>
<td>Hi vs NT</td>
<td>NA</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Hi vs ND</td>
<td>NA</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

NT, nontreated; Low, low-dose therapy; Hi, high-dose therapy; NA, not applicable.

a The sign of the estimate indicates the direction of the comparison between the data sets (i.e., x vs y; positive, x > y; and negative, x < y).

b A mixed-linear model was fit with a variance-covariance structure that incorporated correlation of multiple observations from the same donor.

c Comparisons were analyzed as Poisson distributions of counts using the natural logarithm.

d Nonparametric Wilcoxon sum rank test was utilized since all 20 values of CD34% were equal to 0 for high-dose treatment.

Artificial intelligence facilitated the analysis and summary of this information.
beyond the point of being clonogenic. Since engraftment was low (≤5% human cells) in the transplanted mice that received the high-dose regimen, this may also in part account for the lack of detectable clonogenic cells.

We also looked at the relative contribution of each hematopoietic cell phenotype (CD34, CD19, and CD33) to the human graft in the BM (Table 1 and see supplemental information online). The increasing numerical order for the percentage of CD34+ cells was Hi > Low > NT. There was no significant difference between NT and low-dose cohorts for the percentage of human CD34+ cells (Table 1, \( P > 0.05 \)). As tested by Wilcoxon sum rank test, the percentage of human CD34+ cells was decreased in the high-dose cohort compared to both the low-dose and the NT cohorts (both \( P \) values <0.0001). We observed no significant differences in the percentage of human CD19+ cells between the three experimental groups (Table 1, \( P \) values >0.05). The percentage of human CD33+ cells was significantly increased in both the high-dose and the low-dose treatment groups compared to the NT group (\( P = 0.016 \) and 0.024, respectively). The increasing numerical order for the percentage of human CD33+ cells was NT < Hi < Lo, although high dose and low dose were almost equal and no statistical difference was noted (\( P > 0.05 \)). These data suggest that human myeloid cells that express MGMT^{P140K} were protected more consistently than the other hematopoietic subsets during chemotherapy administration.

Expression of MGMT^{P140K} in the BM of Transplanted Mice Following Delivery of Low- or High-Dose Chemotherapy

Using a fluorometric oligonucleotide assay, we next confirmed that MGMT activity was present in the BM of the transplanted mice following delivery of low- and high-dose therapy (Table 3) [31]. High levels of MGMT activity were present in the marrow of mice transplanted with transduced human MPB following treatment with the low- or high-dose regimens compared to NT and control BM. We observed no substantial differences in the level of MGMT activity in the BM between the low-dose and the high-dose treatments, indicating that high-dose
alkylator treatment did not result in selection or main-
tenance of hematopoietic cells expressing higher levels of
MGMT<sup>P140K</sup> than those mice treated with low-dose
therapy. When using mice that had similar numbers of
huCD45<sup>+</sup>EGFP<sup>+</sup> cells in the BM (Table 3), we did not see a
difference in MGMT DNA-repair activity in mice treated
with low- versus high-dose therapy. However, data
presented here suggest that protection under high-dose
therapy is suboptimal compared to the low-dose treat-
ment in regard to the level of MGMT<sup>P140K</sup>-transduced
human cells following chemotherapy.

We are currently addressing whether low numbers of
primitive human cells that express high levels of
MGMT<sup>P140K</sup> existed after the high-dose therapy and were
either out-competed by the infusion of mouse BM and/or
depleted due to differentiation during the recovery

![FIG. 4. Presence of human CD34<sup>+</sup>EGFP<sup>+</sup> cells following delivery of low- or high-dose 6BG/BCNU. The percentage of human CD34<sup>+</sup>CD38<sup>+</sup> and CD34<sup>+</sup>CD38<sup>-</sup> cells that were EGFP<sup>+</sup> following chemotherapy was assessed by flow cytometry. The percentage of EGFP<sup>+</sup> cells in these populations was determined by setting
gates for human CD34<sup>+</sup>CD38<sup>-</sup> and CD34<sup>+</sup>CD38<sup>+</sup> cells. Representative examples of nontreated (NT), low-dose-treated (Lo), and high-dose-treated (Hi) cells are shown. ND, not detected. The percentage in the upper right quadrant denotes the percentage of the human EGFP<sup>+</sup> cells in the CD34 subset.]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice&lt;sup&gt;a&lt;/sup&gt;</th>
<th>%huCD45&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% huCD45&lt;sup&gt;c&lt;/sup&gt; EGFP&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Total No. human cells (&lt;x10&lt;sup&gt;6&lt;/sup&gt;) plated</th>
<th>Total No. CFU&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Total No. EGFP&lt;sup&gt;f&lt;/sup&gt; CFU&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Total No. EGFP&lt;sup&gt;f&lt;/sup&gt;</th>
<th>% CFU EGFP&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4</td>
<td>40 ± 13.1</td>
<td>0.35 ± 0.2</td>
<td>2.7 (39)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>3599</td>
<td>12</td>
<td>1.5</td>
<td>0.33</td>
</tr>
<tr>
<td>Low&lt;sup&gt;i&lt;/sup&gt;</td>
<td>5</td>
<td>1.3 ± 0.26</td>
<td>71 ± 10</td>
<td>0.2 (51)</td>
<td>928</td>
<td>496</td>
<td>49.6</td>
<td>53</td>
</tr>
<tr>
<td>High&lt;sup&gt;i&lt;/sup&gt;</td>
<td>6</td>
<td>2.1 ± 0.4</td>
<td>99 ± 0.8</td>
<td>0.6 (63)</td>
<td>2</td>
<td>2</td>
<td>0.2</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of mice in which the BM (i.e., femurs) was analyzed by CFU assay. Data were compiled in each treatment group and were derived from MPB 2. MPB 1 was not analyzed for CFU content. Two to four mice per treatment group were analyzed in MPB 3–5 and similar results obtained.

<sup>b</sup> Human cell engraftment in the BM (mean ± SE).

<sup>c</sup> Percentage of human cells that were EGFP<sup>+</sup> in the BM (mean ± SE).

<sup>d</sup> Total number of human CFU detected (BFU-E and CFU-GM combined).

<sup>e</sup> Total number of EGFP<sup>+</sup> human CFU detected in all femurs.

<sup>f</sup> Not treated.

<sup>g</sup> To maximize the number of human cells analyzed, the number of human cells plated per milliliter of complete methylcellulose depended on the level of human cell engraftment in each mouse. Mice with >30% human cell engraftment in the BM were plated at 1 x 10<sup>5</sup> total BM cells, mouse and human, per plate; mice with 10–30% human engraftment were plated at 2 x 10<sup>5</sup> total BM cells per plate and mice with <10% engraftment were plated at 4 x 10<sup>5</sup> total BM cells per plate. (n) is the number of plates analyzed.

<sup>i</sup> Low-dose 6BG/BCNU.
period. The reason for the lack of primitive MPB cells detected in mice receiving the high-dose regimen is most likely the low number of SRC transduced by the oncoretroviral vector. The challenge for future investigations lies in delivering MGMTP140K DNA sequences into sufficient numbers of human HSC derived from MPB that can maintain long-term expression. Cytokine-mediated entry into cell cycle and subsequent breakdown of the nuclear envelope are a prerequisite for integration of oncoretroviral vectors into the host genome. This requirement for provirus integration in conjunction with our previous and current findings may preclude the use of oncoretroviral vectors for gene transfer into MPB. We demonstrated previously that transduction into MPB SRC using oncoretroviral vectors was problematic and that substantial differences in the transducibility of SRC derived from MPB and UCB existed [30]. In this previous study, we showed that although MPB and UCB engrafted

FIG. 5. Effects of low- or high-dose 6BG/BCNU on multilineage engraftment. (A) For B-lymphoid cells, the percentage of EGFP+ cells was determined after gating on human CD34+CD19−, CD34+CD19+, and CD34−CD19+ cells. (B) For myeloid cells, the percentage of EGFP+ cells was determined by setting gates for human CD45+CD33+ cells. Representative examples of nontreated (NT), low-dose treated (Lo), and high-dose treated (Hi) are shown. ND, not detected. The percentage in the upper right quadrant denotes the percentage of the human EGFP+ cells in the denoted subset.
at similar levels in the NOD/SCID mice, transduction efficiency of SRC derived from MPB with oncoretroviral vectors was significantly lower compared to that obtained with SRC derived from UCB. We hypothesize that this difference is due to an overall lack of cytokine responsiveness in MPB SRC compared to UCB SRC and that once MPB SRCs enter the cell cycle, many of them differentiate. In addition, Srour and colleagues demonstrated previously that while UCB SRC in G0 or G1 of the cell cycle engrafted similarly in NOD/SCID mice, MPB SRC in G1 did not engraft as efficiently as MPB SRC in G0 [32,33]. To increase the number of SRCs transduced with the oncoretroviral vector that expresses MGMT\(^{P140K}\), we recently transduced CD34\(^+\) cells isolated from UCB. The striking difference in the transducibility of UCB and MPB in humans awaits the results of clinical gene therapy trials investigating the capability of MGMT\(^{P140K}\)-transduced CD34\(^+\) cells to protect cancer patients during alkylator-based therapy. In summary, these data suggest that improved strategies for transfer of MGMT\(^{P140K}\) into primitive hematopoietic precursor cells to increase DNA repair in hematopoietic cells during high-dose alkylator therapy are needed, particularly when using MPB as a stem cell source. This in vivo model provides an innovative approach with which to determine the impact of high-dose alkylator therapy on human HSC and also to test strategies predicted to increase substantially the number of primitive human hematopoietic cells protected from cytotoxic drug therapy.

**MATERIALS AND METHODS**

**Animals.** A breeding colony of NOD.CB17-Prkdc\(^{scid}\) (NOD/SCID) mice was established at the Laboratory of Animal Research Center at the Indiana University School of Medicine (IUSM) (Indianapolis, IN, USA). All protocols were approved by the Institutional Animal Care and Use Committee. Animals were housed in positive-airflow ventilated racks, bred, and maintained in microisolators under specific-pathogen-free conditions. Following transplantation, animals were placed on static racks and housed under a Biobubble (The Colorado Clean Room Co., Ft. Collins, CO, USA). All mice received 1% neomycin sulfate supplemented with 1.64% glucose in autoclaved water with three changes weekly.

**Retrovirus backbones for expression of MGMT\(^{P140K}\) in HSC.** The oncoretroviral vector SF1-MGMT\(^{P140K}\)-IRES-EGFP (SF1-P140K) was utilized to coexpress MGMT\(^{P140K}\) and EGFP in human CD34\(^+\) cells and was described previously [7]. Retroviral vectors were pseudotyped with the GALV envelope using the PG13 packaging cell line (American Type Culture Collection, Manassas, VA, USA) [35]. Titers were initially determined on human erythroleukemia cells by limiting-dilution analysis. A high-titer clone, SF1-MGMT\(^{P140K}\)-IRES-EGFP (clone 40), produced 2–3 \(\times\) 10\(^5\) infectious units per milliliter and was negative for replication-competent retrovirus.

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<table>
<thead>
<tr>
<th>MPB 5 mouse ID</th>
<th>Treatment</th>
<th>% huCD45(^+)</th>
<th>No. huCD45 per femur ((\times)10(^6))</th>
<th>% huCD45(^+) EGFP(^+)</th>
<th>No. EGFP cells per femur ((\times)10(^6))</th>
<th>MGMT activity (fmol O(^2)-MeG removed/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(^a)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>3072</td>
</tr>
<tr>
<td>Mo 2(^b)</td>
<td>NT(^c)</td>
<td>46.0</td>
<td>3.8</td>
<td>2.2</td>
<td>0.10</td>
<td>14,600</td>
</tr>
<tr>
<td>Mo 4</td>
<td>Low(^d)</td>
<td>5.0</td>
<td>0.1</td>
<td>40.4</td>
<td>0.04</td>
<td>21,800</td>
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<tr>
<td>Mo 6</td>
<td>Low</td>
<td>3.0</td>
<td>0.13</td>
<td>42.5</td>
<td>0.10</td>
<td>28,600</td>
</tr>
<tr>
<td>Mo 8</td>
<td>High(^e)</td>
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<td>0.14</td>
<td>82.5</td>
<td>0.12</td>
<td>26,100</td>
</tr>
<tr>
<td>Mo 9</td>
<td>High</td>
<td>0.7</td>
<td>0.17</td>
<td>88.6</td>
<td>0.15</td>
<td>30,500</td>
</tr>
</tbody>
</table>

\(^a\) Control NOD/SCID BM from a nontransplanted mouse.
\(^b\) Mouse number corresponding to mice in MPB 5.
\(^c\) Not treated.
\(^d\) Low-dose 6BG/BCNU.
\(^e\) High-dose 6BG/BCNU.
Transplantation of NOD/SCID mice. Prior to transplant, NOD/SCID mice were conditioned with 300 cGy total-body irradiation using a GammaCell 40 (Nordion International, Inc., On, Canada) equipped with two opposing 137Cs sources. Cells were washed once and resuspended in IMDM containing 0.2% endotoxin-free BSA and injected into the lateral tail vein of each animal. The number of cells injected per mouse for MBP 1–5 was 28 × 10⁶, 25 × 10⁶, 38 × 10⁶, 45 × 10⁶, and 49 × 10⁶, respectively.

Chemotherapy administration. 6BG (Sigma, St. Louis, MO, USA) was dissolved in 40% polyethylene glycol 400 (v/v) and 60% saline (v/v). BCNU (Sigma) was dissolved in 10% ethanol (v/v) and 90% normal saline solution (v/v). BCNU was placed on ice and used immediately after reconstitution. At 4 weeks posttransplant, cohorts of mice were randomly grouped and either not treated or treated with low- or high-dose chemotherapy according to the schema in Fig. 1. A low-dose cycle consisted of 20 mg/kg 6BG followed by 5 mg/kg BCNU 1 h later. A high-dose cycle consisted of 30 mg/kg 6BG followed by 10 mg/kg BCNU 1 h later and 15 mg/kg 6BG 7 h later. In vivo-selected SFI-P140K-transduced NOD/SCID BM or fresh NOD/SCID BM was infused following high-dose chemotherapy to maintain murine hematopoiesis as detailed under Results and Discussion and Fig. 1A.

Analysis of human cell engraftment. Mice were sacrificed at 8 weeks post-6BG/BCNU injection and single-cell suspensions of the BM were prepared. Human cell engraftment was measured by human CD45 staining and the proportion of engraftment in various lineages was determined by immunostaining and flow-cytometric analysis. Aliquots of 1–2 × 10⁵ cells/tube were stained with various antibodies (2 μl per sample) for 25 min at 4°C in complete medium and washed one time in PBS containing 1% FBS. All antibodies were titrated and used at saturating concentrations. The lack of cross-reactivity of human-specific antibodies with murine cells was confirmed in every experiment by staining BM from a nontransplanted mouse with each antibody combination. Cells were stained with peridinin chlorophyll-conjugated anti-human CD45 (anti-HLe-1; Becton-Dickinson Immunocytometry, San Jose, CA, USA) alone or in combination with phycoerythrin (PE)-conjugated anti-human CD33 (anti-Leu-M9; Becton-Dickinson). Identical aliquots were stained with allopurinol-conjugated anti-human CD34 (clone 581; Pharmingen, San Diego, CA, USA) in combination with anti-human CD19–PE (Pharmingen) or anti-human CD38–PE (anti-Leu-17; Becton-Dickinson). The forward- and right-angle light scatter parameters were used to set the gates for analysis. In experiments in which engraftment of human cells was >5%, ~20,000–40,000 events were collected and analyzed. In experiments in which human engraftment was <5%, ~200,000 events were collected and analyzed. All samples were acquired and analyzed on a Becton–Dickinson FACSCalibur using CellQuest software (Becton–Dickinson).

Statistical analysis. Data were analyzed using SAS version 9 (Cary, NC, USA). Statistical tests were conducted as two-sided at the α = 0.05 significance and 0.10 marginal levels. Pair-wise comparisons between the three groups, nontreated and low-dose and high-dose treatment, were adjusted for multiple comparisons using the Bonferroni method (P < 0.017 for each comparison to maintain overall α of 0.05). The P values for overall significance, difference between groups, and pair-wise comparisons are presented. The percentage of huCD45+, the percentage of huCD45+EGFP+, and the number of huCD45+EGFP+ obtained per femur were analyzed separately. Due to nonconstant variance for each measure, a violation of the assumption of normality, transformations of the data were performed for analysis. A natural logarithm transformation was used for the number of huCD45+EGFP+ and arcsine of the square root transformation was used for proportions of huCD45+ and huCD45+EGFP+. For each analysis, a mixed linear model was fit with a variance–covariance structure that incorporated correlation of multiple observations from the same donor. Typically, samples obtained from the same donor will be more similar than samples obtained from different donors. The percentages of CD319+, CD33+, and CD34+ in the BM were compared separately between the three groups. Except for the percentage of CD34+ cells in the mice treated with high-dose therapy, comparisons were analyzed as Poisson distributions of counts using the natural logarithm of total cells as the offset (count of CD19+ or CD33+ cells of total cells analyzed per sample). Generalized Linear Model analysis was used to account for the correlation between repeated samples from the same donor. Sources of correlation include input percentage of EGFP+ cells, percentage of EGFP+CFU+, total number of cells injected, and number of EGFP+ cells, which are constant for each donor. Nonparametric Wilcoxon sum rank test was used to compare the percentage of CD34+–derived engraftment in the mice treated with high-dose therapy to percentages in the other two treatment groups, since all 20 values of CD34 percentage were equal to 0.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymt.2005.11.017.

REFERENCES