Efficacy and Toxicity of a Virus-directed Enzyme Prodrug Therapy Purging Method: Preclinical Assessment and Application to Bone Marrow Samples from Neuroblastoma Patients

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ABSTRACT

Autologous stem cell transplantation is used to rescue cancer patients from myelosuppression caused by high-dose chemotherapy. However, autologous grafts often contain tumor cells that can contribute directly to relapse. Current purging methods are useful when fewer than 1% tumor cells contaminate the bone marrow, and patients with tumor burdens of >1% are considered ineligible for chemotherapy that necessitates stem cell rescue. Using neuroblastoma (NB) as a model system, we developed a method that is effective even with tumor burdens of 10–25%. Mixtures of NB-1691 NB cells and CD34+ hematopoietic cells purified by this method showed no evidence of viable tumor cells as assessed by clonogenic assays or reverse transcription-PCR for the NB cell markers tyrosine hydroxylase and N-MYC. The efficacy and lack of toxicity of the method were verified using in vivo mouse models. Severe combined immunodeficient mice that received purged cell preparations containing 10% NB-1691 cells survived without evidence of disease for the observation period (>1 year), whereas mice that received unpurged cells developed disseminated disease requiring euthanasia 73–96 days after injection of cells. No evidence of toxicity to the mice was detected by numerous laboratory values for bone marrow, liver, and kidney function, and no difference was seen in the ability of purged cell mixtures versus unmanipulated CD34+ cells to reconstitute the marrow of non-obese diabetic severe combined immunodeficient mice. In a pilot study, marrow was obtained from eight patients who had ≥1% metastatic tumor burden. All eight samples were purged to the level of detection by reverse transcription-PCR (samples from seven patients) or clonogenic potential (sample from one patient), whichever assay was used. The described adenovirus/rabbit carboxylesterase/CPT-11 (irinotecan, 7-ethyl-10-I-(1-piperidino)-1-piperidino)carbonyloxycamptothecin) virus-directed enzyme prodrug method may be useful for patients whose tumor burdens exceed 1% at the time of stem cell harvest. Assessment of purging efficacy with additional samples from NB patients is ongoing.

INTRODUCTION

High-risk NB is the most common indication for autologous stem cell transplantation in pediatric oncology. The use of high-dose chemotherapy or chemoradiotherapy followed by stem cell rescue appears important in improving outcome for patients older than 1 year who have metastatic disease (1–3). Because tumor cells are frequently detected in the blood or bone marrow of patients with advanced NB (4), there is a risk that the patient’s harvested stem cells may be contaminated with tumor. An increasing number of studies shows that up to 50% of bone marrow or peripheral blood stem cell grafts harvested from patients in apparent bone marrow remission contain tumor cells (5–10). Importantly, Rill et al. (11) used gene marking of unpurged bone marrow grafts to demonstrate that contaminating tumor cells can contribute directly to relapse. Effective purging of tumor cells from hematopoietic cells may help optimize treatment and minimize treatment-associated causes of relapse.

Current purging methods for NB use monoclonal antibodies directed against cell surface markers to isolate either tumor cells (12) or hematopoietic stem cells (8, 13). These systems have been shown to reduce the tumor burden of the graft by up to 2–4 logs (8, 12). However, these methods appear to be effective only if the tumor burden at the time of stem cell harvest is less than 1%, and even then, residual tumor cells have been detected after purging (3, 8–10, 12–14). Our goal was to develop a purging protocol that would be effective in reducing the tumor cell number to a level undetectable by RT-PCR even when the tumor burden exceeded 1%.

The VDEPT method described below uses a replication-deficient Ad to deliver the cDNA encoding rCE (AdRSVrCE) selectively to NB cells. rCE efficiently converts the prodrug CPT-11 (irinotecan) to its active metabolite, SN-38 (7-ethyl-10-hydroxy camptothecin). Human tumor cells that express the rabbit CE are up to 500-fold more sensitive to CPT-11 than cells that express only endogenous human CEs (Ref. 15; data not shown), thus potentially providing a favorable therapeutic index. This VDEPT approach has been effective in purging mixtures of 10–25% cultured human NB-1691 NB cells/PBMCNs (16), with a decrease of at least 6 logs of tumor cells. We now describe the purging efficacy and lack of toxicity of the method using an in vivo mouse model of disseminated NB and marrow reconstitution of irradiated NOD/SCID mice and in a pilot study with bone marrow aspirates from eight NB patients.

MATERIALS AND METHODS

NB-1691 Cell Line as a Model System. The NB cell line NB-1691 was obtained from the Pediatric Oncology Group and cultured as reported previously (16). This cell line was chosen as the in vivo model system based on several observations. In a previous study, we determined the sensitivity of seven NB cell lines and primary NB cells to CPT-11 (Ref. 16; data not shown). These seven cell lines were of various genotypes and phenotypes and had either N-myc amplification, N-MYC overexpression, MDM2 amplification, and/or localization of p53 to both nuclei and cytoplasm. All cell lines expressed wild-type p53, as is usually the case clinically. NB-1691 is MDM2 amplified and among the most chemoresistant of the cell lines evaluated. The in vivo model of disseminated disease for this cell line is well characterized (see below). Therefore, the NB-1691 cells represent a stringent test of efficacy of the VDEPT system described and provide a model system with which reproducible, readily interpretable data can be obtained.

CPT-11 was a gift from Dr. J. P. McGovren (Pharmacia Upjohn Co., Kalamazoo, MI). A CPT-11 stock solution of 10 mM was made in 100% DMSO. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
methanol and stored at −20°C for no more than 3 weeks. Dilutions of the stock solution were made with water immediately before use.

Human PBMCs and Human CD34+ Cells. PBMCs were separated from peripheral blood collected from healthy volunteers, using Ficoll-Hypaque (Histopaque-1077; Sigma Diagnostics, St. Louis, MO) according to the manufacturer’s instructions. Granulocyte colony-stimulating factor-mobilized peripheral CD34+ cells were purchased from Poietics of Clonetics (Walkersville, MD). These cell preparations, which contain ~95% human CD34+ cells, were stored in liquid nitrogen until the day of use, when they were thawed quickly and washed with 0.9% NaCl before use.

Generation and Characterization of AdRSVrCE. Construction and characterization of the Ela/I-E3-deleted, replication-deficient Ad containing the RSV promoter and the cDNA encoding a RCE have been described previously (15–19).

The amount of virus used in each experiment is reported as the MOI, which is defined as the number of pfu/total number of cells. The percentage of active virus particles (pfu) compared with total virus particles was ~1%.

Mouse Model of Disseminated NB. The initial description of this model and its recapitulation of the pattern of dissemination of NB in pediatric patients have been published (19). In the initial study, the injection of 5 × 10^6 NB-1691 cells into the tail vein of SCID mice resulted in the death of 100% of mice from disseminated disease in a predictable manner (a median of 42 days). In the current study, various numbers of NB-1691 cells (100 cells to 1 × 10^8 cells) were suspended in sterile saline and injected i.v. into SCID mice. Mice were observed daily, and the day of death was monitored. Animals in obvious distress were euthanized, and the day of sacrifice was recorded as the day of death. The decision to euthanize was made by an animal caregiver having no knowledge of the experimental protocol. Mice were housed and treated in accordance with a protocol approved by the SICRH Committee for Animal Care and Use.

Assessment of Toxicity to Human Hematopoietic Progenitor Cells. A standard in vitro assay was used to assess the ability of human hematopoietic progenitor cells to form colonies in methylcellulose supplemented with growth factors, as published previously (16).

In Vitro Assessment of Cytotoxicity to NB-1691 Cells. Colony-forming assays were performed by standard methods, also as described previously (16).

Assessment of Toxicity to NOD/SCID Marrow Repopulating Cells. NOD/LtSz-Sk/LtSzJ mice were purchased from Jackson Laboratories (Bar Harbor, ME) or were obtained from Dr. Patrick Kelly (SICRH). The ability of human CD34+ cells to repopulate the marrow of NOD/SCID mice was determined using a technique modified from published methods (20–23). All treatments were approved by the SICRH Committee for Animal Care and Use.

Mice (8–12 weeks old) were irradiated with a total of 350 cGy delivered from a 137Cs source at 100 cGy/min, and human CD34+ cells or mixtures of purged NB-1691/CD34+ cells were injected by tail vein 1–3 h after irradiation. In agreement with prior studies, 90%–100% of male and female mice receiving radiation survived the above-mentioned dose of radiation (20). Animals were housed in Barrier III facilities and maintained on standard, nonsterile chow and water. All animals received 50 μl of reconstituted anti-asaialo GM1 (Wako Chemicals, Richmond, VA) on days 1 (the day of irradiation), 4, and 11. NB cells, human hematopoietic cells, and mixtures of these cell types were suspended in PBS at concentrations such that 150 μl of cell suspension contained the number of cells to be injected.

SCID mice were used to assess purging efficacy (the method described above), and NOD/SCID mice were used to assess toxicity to marrow repopulating cells because NB-1691 cells do not form tumors in NOD/SCID mice.

Toxicity Testing after Irradiation and Marrow Engraftment. Toxicity testing was done 1, 4, and 8 weeks after injection of human cells. Peripheral blood was collected from each mouse via the retro-orbital sinus, using a narrow bore glass Pasteur pipette. Blood for a complete blood count was collected in EDTA. Complete blood counts were done using a Hemavet 5700 Multispecies Hematology Analyzer (CDC Technologies, Oxford, CT). This analyzer provides a five-part differential (segmented neutrophils, lymphocytes, monocytes, basophils, and eosinophils), as well as other blood indices including total WBC, RBC Hgb, hematocrit, mean corpuscular volume, mean corpuscular Hgb, mean corpuscular Hgb concentration, and platelets. Reticulocyte smears were prepared in the St. Jude Animal Resource Center Diagnostic Laboratory and sent to Ani-Lytic, Inc. (Guthersburg, MD) for interpretation. Reticulocyte smears were prepared by mixing equal volumes of EDTA blood and New Methylene Blue N. The mixture was allowed to stand for 30 min at room temperature and then smeared onto a glass slide.

Blood for serum chemistry analysis was collected without anticoagulant and allowed to clot. The clot was removed from the serum, and serum chemistry analysis was performed using a VetScan Chemistry Analyzer (Abaxis, Inc., Sunnyvale, CA), which provides a diagnostic panel that includes albumin, alkaline phosphatase, ALT, amylase, TBIL, blood urea nitrogen, calcium (Ca2+), total cholesterol, creatinine, glucose, potassium (K+) total protein, and globulin. Other chemistry tests such as those for AST, γ-glutamyl transferase, sodium, phosphorus, chloride, protein electrophoresis, and triglycerides were prepared and sent to Ani-Lytics, Inc. for analysis.

At the end of each experiment (8 week time point), each mouse was anesthetized using an admixture of ketamine (100 mg/ml) and xylazine (20 mg/ml) administered i.p. at a dose of 0.2 ml/mouse. Each mouse was exsanguinated, and the blood was collected according to the procedures described above. After exsanguination, liver, lung, spleen, gonads, and bone marrow were collected from each mouse. All tissues were submitted to the St. Jude Animal Resource Center Histology Laboratory for routine H&E staining. A partial listing of results obtained from the above-mentioned tests is included in this article.

Immunohistochemical Staining of Human CD45+ Cells in Mouse Marrow. Antihuman CD45+ conjugated to PE was purchased from BD Phar-Mingen (San Diego, CA). PE-conjugated isotype-matched mouse immunoglobulin was used as a negative control. Immunofluorescence staining was done by the method reported previously (24), except that after harvesting marrow from both femurs of each mouse and washing of cells with PBS, immunostaining was done on unfixed cells. All reagents were used and procedures were done from light during staining and analysis.

Flow Cytometric Analysis. Samples (5 × 10^6) from unmanipulated mice, mice receiving control CD34+ cells, or mice receiving purged samples of 10% NB-1691/90% CD34+ cells were resuspended in 0.5 ml of saline and counterstained with PI. The percentage of human CD45+ cells was quantitated using a fluorescein-activated cell-sorting Calibur flow cytometer (Becton Dickinson, San Jose, CA) by collecting PE fluorescence at 585 ± 21 nm and PI fluorescence at wavelengths greater than 670 nm. Viable cells were selected by electronic gating for single cells with low PI fluorescence. The percentage of viable cells with positive levels of PE fluorescence (human CD45+) was determined by comparison with background levels of PE fluorescence in control samples containing cells from normal mouse marrow.

Purging and Assessment of Purging Efficacy and Toxicity in Preclinical Models. Human hematopoietic cells or mixtures of NB/hematopoietic cells (1–2 × 10^7, unless otherwise indicated) were placed in culture (see figures above). For high-risk NB, polymorphonuclear cells were isolated by Ficoll separation (16).

Nucleated cells were then processed according to the described purging protocol. Bone marrow biopsies obtained at the same time as the aspirates were reviewed by the attending pathologist, who estimated the percentage of tumor
burden by conventional light microscopy. Whereas microscopic evaluation of tumor burden is not quantitative, and methods such as real-time RT-PCR for tumor cell markers may eventually replace the current standard methods, microscopic estimates of tumor burden are included (Fig. 5) as the current standard to which other methods are compared. Informed consent was obtained from all patients according to institutional guidelines.

**Assessment of Viable NB Cells by RT-PCR after Purging.** Total RNA was extracted from samples using an RNAqueous nucleic acid extraction kit (Ambion, Inc., Austin, TX). Two μg of RNA were reverse-transcribed using Ready-to-Go You-Prime First-Strand beads (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). PCR analysis was done for two specific NB markers: TH and N-MYC. Primers to detect β-actin were used to verify the integrity of the RNA and served as a positive control for the RT-PCR reactions. A no-template water control was used as a negative control. The primer sequences and PCR conditions have been previously described (16). Each set of primers spanned an intron to eliminate signal that might be contributed by low levels of genomic DNA in the RNA preparations. RT-PCR products were separated by agarose gel electrophoresis.

**Statistical Analysis.** Data in Table 1 were analyzed using a one-way ANOVA with a Bonferroni post test. 

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**RESULTS**

**In Vivo Assessment of Purging Efficacy**

A previous study (16) from our laboratory evaluated the effect of AdRSVrCE or CPT-11 alone and in combination on the clonogenic survival of NB cells. Data with NB cell lines of various phenotypes showed that 5 μM CPT-11, which was the highest dose that was relatively nontoxic to hematopoietic cells, reduced the clonogenic potential of NB cell lines by ~25–50%. AdRSVrCE alone (100 MOI) decreased the clonogenic potential of NB cell lines by ~10–50%. These data documented that AdRSVrCE or CPT-11 alone was insufficient to eradicate the clonogenic potential NB cells.

We also demonstrated previously by two in vitro assays, clonogenic potential and RT-PCR, that the AdRSVrCE/CPT-11 VDEPT method effectively reduced the number of NB cells in a 10% NB-1691/90% PBMC mixture to undetectable levels (15, 16). The next step in assessing the utility of this method for eventual clinical application was to evaluate the efficacy and potential toxicities of the approach in in vivo mouse models.

Experiments to assess purging efficacy were done by mixing 10% NB-1691 cells with 90% human PBMCs and injecting either unpurged or unpurged mixtures i.v. into the tail vein of SCID mice. Because i.v. injection of ~10⁷ NB-1691 cells produces disseminated disease in 100% of SCID mice (19), we anticipated that all of the mice injected with unpurged cell preparations would develop disseminated disease. However, the sensitivity and reliability of this model in detecting the small numbers of viable NB cells that might remain after purging were unknown. Therefore, we first determined the number of NB-1691 cells required to produce disseminated disease in this SCID mouse model. Fig. 1a shows survival curves for five groups of SCID mice injected with the indicated number of NB-1691 tumor cells. The data demonstrate that infusion of ~10⁶ NB cells produced disseminated disease and death, in a dose-dependent manner. Mice receiving only 100 NB-1691 cells showed no evidence of disease for ~1 year. Mice receiving 10⁷ or 10⁸ cells developed advanced disease requiring euthanasia 73–96 days after injection. The results show that this in vivo assay is capable of detecting 1000 viable NB-1691 cells. The data also suggest that even in immune-compromised SCID mice, there is a level of NB cells that is tolerated and does not result in systemic disease.

To then test the efficacy of the rCE/CPT-11 VDEPT purging protocol using this model, we mixed 10% NB-1691 cells/90% PBMCs and exposed samples to AdRSVrCE and CPT-11 as described. Purged or control mixtures of cells containing 10⁵ NB-1691 cells in a total of 10⁶ cells were injected i.v. into each of 14 mice/group. Fig. 1b shows survival curves for SCID mice that received i.v. injection of either purged or unpurged samples. All 14 mice receiving unpurged samples developed disseminated NB that required euthanasia 73–96 days after injection. Conversely, all 14 mice receiving
VDEPT FOR PURGING NEUROBLASTOMA CELLS

Assessment of Purging Toxicity

We assessed the toxicity of purging to CD34<sup>+</sup> cells by performing marrow reconstitution experiments in irradiated NOD/SCID mice. Results are expressed as the percentage of human hematopoietic cells (human common leukocyte antigen CD45<sup>+</sup> cells) in the mouse marrow 8 weeks after injection of purged cell mixtures or CD34<sup>+</sup> cells. Fig. 3 shows the percentage of human CD45<sup>+</sup>/total nucleated cells in mouse marrow from mice receiving purged NB/CD34<sup>+</sup> cell mixtures versus unmanipulated CD34<sup>+</sup> cells. In the group of mice receiving 3 × 10<sup>6</sup> unmanipulated CD34<sup>+</sup> cells (control mice), one of nine mice failed to engraft (indicated by the filled diamond). In the eight control animals that did engraft, the percentage of human CD45<sup>+</sup> cells in the marrow ranged from 0.60% to 5.28%. For mice receiving mixtures of NB/CD34<sup>+</sup> cells that had been exposed to a viral MOI of 50 and a CPT-11 concentration of 5 μM, eight of eight mice engrafted, with percentages of human cells in the marrow of 1.28–10.75%. Similarly, in the group of mice receiving cells purged with an adenoviral MOI of 100 plus 5 μM CPT-11, all 10 mice reconstituted their marrow with percentages of human cells ranging from 0.36% to 16.92%. There was no statistical difference in the percentage of human cells in the marrows of the three groups of mice. We conclude that the purging with adenoviral MOIs of 50 and 100 produced no detectable toxicity to human CD34<sup>+</sup> NOD/SCID reconstituting cells.

Assessment of Effects on Mouse Organ Function

Laboratory measures of hepatic and renal function of mice injected with unmanipulated CD34<sup>+</sup> cells or purged CD34<sup>+</sup>/NB cells were obtained. Each group of mice comprised 8–12 animals, and values were obtained from all or a subset of animals in each group at 1, 4, and 8 weeks after injection of CD34<sup>+</sup> cells or purified CD34<sup>+</sup>/NB cells. Table 1 reports the mean values ± SD for the week 4 time point. No significant differences were seen in values obtained from samples of mice receiving unmanipulated CD34<sup>+</sup> cells compared with purged cell mixtures (Table 1). Furthermore, multiple statistical analyses comparing values obtained at the three different time points for each group of mice or at a single time point for all three groups of mice failed to show any differences between mice that received unmanipulated CD34<sup>+</sup> cells and irradiated, “transplanted” mice that received purged 10% NB/90% CD34<sup>+</sup> cell mixtures (data not shown). We did observe several individual mice in each group with AST levels higher than the normal range, and these values are reflected by the relatively large SD for AST levels reported in Table 1. However, each of these individual mice had normal ALT and TBil levels, verifying that liver function in these mice was within normal range. We conclude that liver and kidney function in all of the mice was within normal limits.

Materials and Methods

"Materials and Methods."
transcription reaction and positive controls in which primers for
shown is a negative control in which no RNA was used in the reverse
visualized by UV light. Procedures were done by standard techniques; primer sequences
sis, the gel was stained with ethidium bromide, and the products of the reaction were
for TH and β-actin. RT-PCR products were separated by agarose gel electrophoresis, the gel was stained with ethidium bromide, and the products of the reaction were visualized by UV light. Procedures were done by standard techniques; primer sequences and annealing temperatures for RT-PCR have been published previously [16].

**Assessment of Toxicity and Efficacy of Purging on Patient Bone Marrow Samples by RT-PCR and Clonogenic Assay**

**Documentation of the Sensitivity of RT-PCR to Detect NB-1691 Cells.** To assess the ability of RT-PCR to detect low numbers of NB cells, we made 10-fold dilutions ranging from 1 NB-1691 cell in $10^3$ PBMCs to 1 NB cell in $10^7$ PBMCs and extracted RNA from a total of $5 \times 10^7$ cells of each mixture. RT-PCR products for TH and N-MYC obtained with RNA extracted from dilutions of $1:10^3$ to $1:10^7$ for TH and $1:10^6$ and $1:10^7$ for N-MYC are shown in Fig. 4. Also shown is a negative control in which no RNA was used in the reverse transcription reaction and positive controls in which primers for β-actin were used to verify the integrity of the RNA used in the reaction. Whereas the sensitivity of RT-PCR depends on the level of expression of a given transcript in a specific cell line or tumor, the data show that when NB-1691 cells are used as the “standard,” the message for TH is detectable at a level of $1:10^3$ cells, and the message for N-MYC is detectable at a level of $1:10^6$ or $1:10^7$ cells. Results shown are from one of five replicate experiments. Results among the five experiments were reproducible, with the exception of some variability as to whether N-MYC transcript was/was not detected at a dilution of $1:10^7$ cells, suggesting that this concentration is at the borderline of detection.

**Pilot Purging Study with Samples from Four NB Patients.** For this pilot study, we performed the purging procedure on aliquots of bone marrow aspirated from eight patients with metastatic NB. Samples were divided into aliquots of approximately $2–7.5 \times 10^7$ cells each. Purging was performed using a viral MOI of 50 or 100 and a CPT-11 concentration of 5 μM. For each sample, aliquots containing the same number of cells were maintained in culture as untreated controls. RNA from both purged and unpurged samples was harvested 7–8 days after exposure of the purged aliquot to CPT-11.

Fig. 5 shows primary data from four of the patient samples. RT-PCR using RNA from bone marrow samples obtained from three of the patients (Fig. 5a) detected N-MYC transcripts in the unpurged aliquots, but not in the purged aliquots. Two of these three tumors also expressed TH as shown by the RT-PCR product in the unpurged aliquots, but no TH signal was present in the purified samples. No signal was detected in the control reaction to which no RNA was added (data not shown). The attending pathologist reviewed bone marrow biopsies obtained on the same day as the aspirates and estimated the tumor burden by conventional light microscopy for the three patients to be 15%, 2%, and 1%, as indicated.

Fig. 5b shows results from a “clonogenic assay” with a fourth sample obtained from a patient who had relapsed after treatment with stem cell transplant. The bone marrow biopsy showed nearly complete replacement of the marrow space by metastatic tumor cells. Therefore, beginning with a total cell number of $2 \times 10^7$, if purging was successful, there would have been an insufficient number of cells remaining to extract the 2 μg of RNA required for RT-PCR. Instead, purged and unpurged aliquots of this sample were plated and maintained in culture for 8 weeks. Unpurged control samples grew readily in culture; no cells were detected in the flask containing purged cells (Fig. 5b). Cells in the unpurged flask expressed N-MYC and TH (data not shown).

A summary of purging results from all eight patients is shown in Table 2. Seven of the eight clinical samples were completely purged using the AdRSVrCE virus to the level of RT-PCR detection. The purge failure (patient 8) was likely due to a low level of rCE expression (data not shown). We have subsequently used a slightly modified Ad to successfully purge a sample obtained from patient 8 (see “Discussion”).

Additionally, there was a sufficient number of cells available from patient 2 (10–15% tumor burden) to do an *in vitro* progenitor cell assay to assess the toxicity of the purging protocol. Purging was performed with a viral MOI of 50 and 5 μM CPT-11. Multiple plates (7–10 plates) were seeded for control and purged samples. The average ± SD number of progenitor cell colonies growing in methylcellulose 12 days after plating of the cells was 90 ± 24 and 85 ± 37 for unpurged cells and purged cells, respectively. These data indicate that the rCE/CPT-11 VDEPT purging protocol did not affect the ability of hematopoietic progenitor cells to form colonies in methylcellulose, using cells from a bone marrow aspirate of a patient with NB. The data indicate that further evaluation of rCE/CPT-11 VDEPT for purging is warranted.
in treatment for metastatic disease. Samples were purged according to the protocol described elsewhere (12, 31). Also, up to 50% of stem cell grafts collected from NB patients in whom purge failure seen thus far, was successfully purged using an AdCMVrCE construct (data not shown). Further evaluation of additional constructs is ongoing.

DISCUSSION

Whereas VDEPT approaches to stem cell purging have been reported for adult malignancies such as breast cancer (25) and multiple myeloma (26), our laboratory is the first to describe its use for NB (15) and to investigate the novel enzyme/prodrug combination rCE/CPT-11 for potential clinical application. Perhaps most importantly, this study illustrates an attempt to optimize each component of a gene therapy approach to achieve a specific therapeutic goal. It is likely that gene-based therapies will have significant clinical impact only when each component has been optimized.

The rationale for the specific application of rCE/CPT-11 for purging NB cells was based on several observations. First, at the MOI used, the Ad specifically transduces NB cells compared with hematopoietic cells (16). Second, overexpression of rCE sensitizes cells that express endogenous levels of human CEs to CPT-11 by up to 500-fold (15). Third, CPT-11 has demonstrated significant, dose-dependent antitumor activity against NB xenografts (27), and clinical responses have been seen in patients with high-risk NB in a Phase I trial (28). These studies suggest that the high intracellular levels of SN-38 achievable with rCE/CPT-11 VDEPT may produce effective NB cell kill, and results with clinical specimens (Fig. 5 and Table 2) are encouraging. However, because one of eight samples failed to purge with AdRSVrCE, we have begun to evaluate additional viral constructs, and we have identified promoters that produce higher levels of rCE in both NB cell lines and primary tumor cells than the RSV promoter. Interestingly, a sample from patient 8, the single purge failure seen thus far, was successfully purged using an AdCMVrCE construct (data not shown). Further evaluation of additional constructs is ongoing.

Autologous stem cell transplantation is routinely used to treat high-risk NB and appears important in improving disease-free survival. Controlled studies to assess the benefit of purging in NB have not yet been completed; it is not known whether purging is essential in the treatment of this disease, but several observations suggest that purging provides some clinical benefit. Adult lymphoma patients who receive stem cell grafts free of residual disease show improved survival compared with patients who receive contaminated grafts (29–31). Also, up to 50% of stem cell grafts collected from NB patients in apparent remission are contaminated by tumor cells that can be tumorigenic (32) and contribute to relapse (11). Thus, for patients with bone marrow involvement, purging is likely an important component of successful therapy, and it has become part of many cooperative group treatment protocols (1, 3, 14, 33).

In addition to reducing the likelihood of tumor cell reinfusion, more effective purging methods may also offer other advantages. Improved purging efficacy may make possible the option of earlier stem cell harvesting, resulting in more efficient collection of stem cells (34) and more rapid engraftment (35). Earlier harvest may protect stem cells from certain deleterious effects of chemotherapy such as secondary leukemias, which occur in up to 7% of children receiving contemporary regimens for high-risk NB (36).

The described rCE/CPT-11 VDEPT method can be performed with either bone marrow grafts or peripheral blood stem cells and appears to be feasible as well as relatively effective and nontoxic. Also, in contrast to currently available purging methods, the described VDEPT approach is efficacious with tumor burdens estimated to be greater than 1%. Whereas we do not envision that stem cell harvest would be done when patients have 25% NB cells in their marrow, a method such as rCE/CPT-11 VDEPT may have utility in patients whose marrow contains ~1–5% tumor cells (because these patients are not eligible for standard purging protocols) or when early harvest would likely yield more viable stem cells.

Laboratory manipulations involve only maintaining the cells in culture for a total of 76 h and exposing the cells to virus and CPT-11 during this time. We did note that the apparent viability of NOD/SCID repopulating cells that have been maintained in culture for 76 h, even with recommended concentrations of specific growth factors, may be lower than that of CD34+ cells that have been stored at ~80°C until the day of use. Accordingly, the NOD/SCID engraftment experiments (Fig. 3) were done by injecting 3 × 10^6 viable CD34+ cells/mouse (plus or minus NB cells and purging). This number of cells was chosen because it is the lowest number of peripheral CD34+ cells that can reproducibly repopulate the marrow of NOD/SCID mice that receive 350 cGy of radiation (20). Therefore, if the purging procedure was toxic to the subset of cells responsible for marrow reconstitution in the mice, engraftment failures in the mice receiving purged cells compared with unmanipulated cells should have been detectable. No such engraftment failures were seen. One hundred percent of mice receiving unpurged cells engrafted, compared with 89% of the mice receiving unpurged CD34+ cells. However, it should be noted that in preliminary experiments (data not shown) in which we injected 3 × 10^6 CD34+ cells that had never been maintained in culture ex vivo into each of six mice, the percentage of CD45+ human leukocytes in the marrow of those mice ranged from 7.41% to 67.4%. It is controversial whether the precise percentage of CD45+ cells detected in mouse marrow is a more meaningful parameter than the presence versus absence of human CD45+ cells (20, 21) because few mice receiving these low levels of peripheral blood CD34+ cells have been studied. We conclude that whereas the purging protocol itself is not toxic to NOD/SCID repopulating cells, and 100% of transplanted mice engrafted with human hematopoietic cells, there may be some decrease in the percentage of human cells in the NOD/SCID marrow 8 weeks after cell infusion if CD34+ cells are maintained ex vivo for 3 days before injection into the mice. The relevance of this observation as it might apply to autologous stem cell rescue is unknown.

In conclusion, we describe an approach to stem cell purging for NB that may decrease tumor cell contamination of hematopoietic cell grafts to levels below those detectable by RT-PCR, even when the initial level of tumor cell contamination exceeds 1%. Such purging efficacy may not only reduce the likelihood of tumor cell reinfusion but may also allow administration of high-dose consolidation therapy to more patients in a more timely manner. Although additional innovative therapies may ultimately be required to cure high-risk patients, this purging method may help optimize existing therapies. We are evaluating additional patient samples to assess the efficacy of the purging protocol for a variety of NB patients. If we continue to observe the efficacy seen with samples in the pilot study, a clinical trial will be proposed for patients with levels of bone marrow contamination above 1%.

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Footnotes

1 M. K. Danks and P. M. Potter, unpublished observations.

Table 2 Summary of results with clinical samples in pilot purging study

Bone marrow aspirates containing NB cells were obtained from patients undergoing treatment for metastatic disease. Samples were purged according to the protocol described in “Materials and Methods.”

<table>
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<th>Patient no.</th>
<th>Estimated tumor burden</th>
<th>P/F</th>
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<td>1%</td>
<td>P</td>
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<tr>
<td>2</td>
<td>10–15%</td>
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</tr>
<tr>
<td>7</td>
<td>15–40%</td>
<td>P</td>
</tr>
<tr>
<td>8</td>
<td>&lt;5%</td>
<td>F</td>
</tr>
</tbody>
</table>

* P, purged; F, failed purge. Status was determined by RT-PCR for TH and N-myc.

**Determined by clonogenic assay.

See “Discussion.”

**
disease greater than 1% for whom conventional purging would be inappropriate.

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Efficacy and Toxicity of a Virus-directed Enzyme Prodrug Therapy Purging Method: Preclinical Assessment and Application to Bone Marrow Samples from Neuroblastoma Patients

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