A Virus-directed Enzyme Prodrug Therapy Approach to Purging Neuroblastoma Cells from Hematopoietic Cells Using Adenovirus Encoding Rabbit Carboxylesterase and CPT-11

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ABSTRACT

Tumor cells that contaminate hematopoietic cell preparations contribute to the relapse of neuroblastoma patients who receive autologous stem cell rescue as a component of therapy. Therefore, effective purging methods are needed. This study details in vitro experiments to develop a viral-directed enzyme prodrug purging method that specifically targets neuroblastoma cells. The approach uses an adenovirus to deliver the cDNA encoding a rabbit liver carboxylesterase that efficiently activates the prodrug irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy camptothecin (CPT-11). The data show that an adenoviral multiplicity of infection of 50 transduces 100% of cultured neuroblastoma cells and primary tumor cells, irrespective of the level of tumor cell line contamination. Exposure of neuroblastoma cell lines or of mixtures of these cell lines with CD34+ cells at a ratio of 10:90 to replication-deficient AdRSVrCE for 24 h and subsequent exposure of cells to 1–5 μM CPT-11 for 4 h increased the toxicity of CPT-11 to three neuroblastoma cell lines (SJNB-1, NB-1691, and SK-N-SH) from ~20–50-fold and eradicated their clonogenic potential. Also, after “purging,” RNA for neuroblastoma cell markers (tyrosine hydroxylase, synaptophysin, and N-MYC) was undetectable by reverse transcription-PCR. In contrast, the purging protocol did not affect the number or type of colonies formed by CD34+ cells in an in vitro progenitor cell assay. No bystander effect on CD34+ cells was observed. The method described is being investigated for its potential clinical utility, particularly its efficacy for use with patients having relatively high tumor burdens, because no published methods have been shown to be efficacious when the tumor burden exceeds 1%.

INTRODUCTION

Despite improved cure rates for other pediatric cancers, long-term survival for high-risk metastatic NB remains poor (1, 2). Dose-intensive chemotherapy regimens have increased the likelihood of attaining partial and complete responses (3), but many of these patients will relapse and ultimately succumb to their disease. New approaches such as 13-cis-retinoic acid (4), anti-GD2 antibody (5), and metadiobenzylguanidine delivery of radioisotopes (6) look promising but have been used thus far only as adjuncts to high dose chemotherapy and hematopoietic stem cell rescue. Myeloablative chemotherapy followed by autologous stem cell rescue is routinely used for high-risk patients (7), but gene-marking studies (8) have shown that contaminating tumor cells contribute to relapse after transplant for NB. Therefore, effective methods are needed for purging NB cells from bone marrow or peripheral stem cells before reinfusion.

Purging techniques target exploitable differences between tumor cells and hematopoietic cells to produce tumor cell-specific depletion. A variety of purging methods have been reported. Shpall et al. (9) purged breast cancer cells from hematopoietic cells using immunomagnetic techniques. Kies et al. (10) used a discontinuous bovine albumin gradient to select out hematopoietic progenitor cells in bone marrow samples contaminated with breast cancer cells. Stribbling et al. (11) attached prodrug-activating enzymes to tumor-specific antibodies (antibody-directed enzyme prodrug therapy) to produce tumor cell-selective drug activation and toxicity. A recently published clinical study (12) used a combination of sedimentation, filtration, and magnetic immunobeads for separation of tumor cells from hematopoietic cells before autologous stem cell rescue of NB patients. One of the eligibility criteria for this trial was a tumor burden at harvest of ≤1% NB cells.

Of the reported approaches, VDEPT using adenoviral vectors seems particularly promising (13–15). Clarke et al. (16) first reported the selective transduction by Ad of NB cells and breast cancer cells compared with hematopoietic cells. Chen et al. (17) determined that this tumor cell selectivity was likely explained by the presence or absence of the coxsackie Ad receptor needed for binding of Ad to the cell surface and expression of αvβ3 or αvβ5 integrins required for internalization of the virus into the cell. The premise that underlies VDEPT approaches to purging using adenoviral vectors is that Ades achieve tumor cell-specific delivery and expression of a cDNA that encodes a drug-activating enzyme, and subsequent exposure to the appropriate prodrug results in activation of the prodrug selectively in tumor cells expressing the transgene.

The best characterized VDEPT purging approaches use a replication-deficient Ad to deliver the cDNA encoding Hsvtk to sensitize tumor cells to ganciclovir (14–15). Using Ad, Hsvtk, and ganciclovir, Teoh et al. (14) eradicated multiple myeloma cells without affecting the viability of hematopoietic progenitor cells. In other studies (15) in which similar methods were used, however, two to six log reductions of tumor cells were demonstrated, but viable tumor cells remained. Likely, the clinical potential of VDEPT will be achieved by optimizing each component (virus, enzyme, and prodrug) to the specific tumor being targeted.

We are investigating a VDEPT approach designed to purge NB cells from hematopoietic stem cells using Ad, rCE, and CPT-11. Ad selectively transduces NB cells; overexpression of rCE sensitizes tumor cells to CPT-11 (18–21); and NB tumors are relatively sensitive to SN-38, the active form of CPT-11 (22, 23). The following study describes in vitro experiments to develop this VDEPT approach to purging.

MATERIALS AND METHODS

Cell Lines and Drugs

The NB cell lines SK-N-SH, SK-N-AS, and IMR32 were obtained from American Type Culture Collection (Rockville, MD). The NB cell line SJNB-1 was obtained from the American Type Culture Collection (Rockville, MD). The NB cell line SJNB-1 contains a single retroviral integration as determined by Southern blot and PCR. The NB cell line SK-N-SH contains a single retroviral integration as determined by Southern blot and PCR. The NB cell line SK-N-AS contains a single retroviral integration as determined by Southern blot and PCR. The NB cell line IMR32 contains a single retroviral integration as determined by Southern blot and PCR. The NB cell line SJNB-1 contains a single retroviral integration as determined by Southern blot and PCR.

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The abbreviations used are: NB, neuroblastoma; Ad, adenovirus; β-gal, β-galactosidase; CE, carboxylesterase; CPT-11, irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy camptothecin; Hsvtk, Herpes simplex virus thymidine kinase; MOI, multiplicity of infection; PBMC, peripheral blood mononuclear cell; rCE, rabbit liver CE; SCF, stem cell factor; SN-38, 7-ethyl-10-hydroxy camptothecin; SYN, synaptophysin; TH, tyrosine hydroxylase; VDEPT, virus directed enzyme prodrug therapy; X-gal, 5-bromo-4-choro-3-indolyl-β-d-galacto- side; IL, interleukin; RT-PCR, reverse transcription-PCR; BA, β-actin; RSV, Retro sarcoma virus.

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was established at St. Jude Children’s Research Hospital, in accordance with the guidelines of the Institutional Review Board. NB-1691 and NB-1643 cell lines were obtained from the Pediatric Oncology Group. Cell lines were grown in DMEM (SK-N-SH, SK-N-AS, and IMR32) or RPMI 1641 medium (NB-1691, NB-1643, and SJNB-1) supplemented with 10% fetal bovine serum and 2 mM l-glutamine.

CPT-11 stock solution (10 mM) in methanol was stored at −20°C, and dilutions were made with water immediately before use.

Human Peripheral Mononuclear and CD34⁺ Cell Preparations

Peripheral blood was collected from healthy volunteers, and the PBMCs were harvested using Ficoll-Hypaque (Histopaque-1077) according to the directions of the manufacturer (Sigma Chemical Co. Diagnostics, St. Louis, MO). PBMCNs were used the same day they were collected. Granulocyte-stimulating factor-mobilized CD34⁺ peripheral blood cells were purchased from Poietics of Clonetics (Walkersville, MD) and stored in liquid nitrogen. On the day of use, CD34⁺ cells were thawed quickly, pelleted by centrifugation, and washed once with 0.9% NaCl.

Viral Transduction Efficiency

An E1a-, E3-deleted, replication-deficient Ad containing the RSV promoter and the reporter gene β-gal (AdRSV β-gal) was obtained from Genetic Therapy, Inc., a Novartis company (Gaithersburg, MD). This Ad was used to assess the transduction efficiency of Ad for cell lines, primary NB, and hematopoietic cells. Cells were plated at a concentration of 40,000 cells/well on 2-well chamber slides (LAB-TEK, Naperville, IL) and exposed to virus in 2% or 10% serum at MOIs ranging from 1–500 for 24 h. The cells were then incubated an additional 24 h to allow for protein expression and fixed in 2% paraformaldehyde/0.2% glutaraldehyde in PBS, washed in PBS, and incubated overnight with X-gal substrate (24). To determine the percentage of cells transduced, 200 cells from each chamber were counted, and the number of positively stained cells was noted. A cell was considered positive only if it appeared very dark blue. Each MOI determination was done in triplicate, and slides were read by two investigators independently. PBMCNs were also assayed for β-gal activity as above with the following modification; after the 24-h incubation to allow for protein expression, the suspension of PBMCNs was fixed onto glass slides using a Shandon CytoSpin 3 cytocentrifuge at 400 rpm for 8 min.

Assessment of Cytotoxicity to Nb Cell Lines

To determine the effect of virus, CPT-11, or the combination on NB cell lines, clonogenic assays were performed. Cells (3000/well) were plated in 6-well plates (Costar, Cambridge, MA), allowed to attach, and then exposed to virus, drug, or both. The toxicity of viral MOIs of 1–100 for 24 h and various concentrations of CPT-11 for 4 h were assessed. The combined toxicity of Ad and CPT-11 was evaluated by exposing the cells to virus for 24 h and then adding CPT-11 for 4 h. 48 h after virus had been removed. After a time equivalent to five doublings of the untreated control cells, the cells were stained with crystal violet, and colonies of >10 cells were imaged using the Alpha Imager 200 documentation system (Alpha Innotech Corporation, San Leandro, CA) and counted using Labworks computer software by UVP Technologies. Results are expressed as the percentage of survival compared with that of untreated control colonies. Dishes were also checked microscopically to verify results when no colonies were detected by the automated counter.

Assessment of Cytotoxicity to Human Progenitor Cells

To evaluate the effect of Ad, CPT-11, or both on hematopoietic cells, methylcellulose-based assays (25, 26) were performed on PBMCNs and on granulocyte-stimulating factor-mobilized peripheral CD34⁺ cells.

PBMCNs. After Ficoll-Hypaque separation, the mononuclear cell layer of peripheral blood was obtained, and the number of nucleated cells was counted using a hemocytometer. PBMCNs (1 × 10⁶) were aliquoted into 35-mm cell suspension dishes (Sarstedt, Newton, NC) and exposed to various MOIs of Ad for 24 h, to CPT-11 for 4 h, or to both. After exposure to virus with or without CPT-11, adherent cells were dislodged, and cell suspensions were transferred to microcentrifuge tubes and centrifuged at 2000 rpm for 5 min. Supernatants were discarded, and the cells were resuspended in 500 μl of 2% Iscove’s modified Dulbecco’s medium. The 500 μl of cell suspension was then added to 5 ml of Methocult GF H4434 containing 50 ng/ml recombinant human SCF, 10 ng/ml IL-3, and 3 units/ml erythropoietin (Stem Cell Technologies, Vancouver, British Columbia, Canada) and vortexed. Aliquots (1.2 ml) were distributed by syringe with a blunt-end needle into 35-mm gridded dishes (NUNC, Naperville, IL). Dishes were incubated at 37°C in high humidity, and colonies were counted microscopically between day 10 and day 14. Results are reported as the total number of colony forming units for neutrophils and monocytes, colony-forming units for late erythroid progenitors, and colony-forming units for granulocytes, erythrocytes, macrophages, and megakaryocytes, compared with untreated controls.

CD34⁺ Cells. CD34⁺ cells were processed as the PBMCNs were except that cells were plated at 1200 cells/dish. It should be noted that when the above concentrations of SCF, IL-3, and erythropoietin are used to culture CD34⁺ cells in vitro, the cell number may increase by ~1.2-fold to ~1.9-fold in 2–4 days. Therefore, the percentage or number of colonies surviving exposure to Ad or CPT-11 reflects any increase in cell number occurring during that time, combined with the effect of Ad and/or drug treatment on the cells originally plated.

Quantitation of CE Activity

CE activity was determined as described previously (18). One unit of activity is defined as μmol of o-nitrophenol produced from o-nitrophenyl acetate/mg protein/min.

Purging of NB Cell Lines from PBMCNs or CD34⁺ Cells

AdRSvRCE Virus. A replication-deficient E1a-, E3-deleted Ad containing the cDNA encoding an intracellular form of rCE was used for purging experiments. Expression of the CE was regulated by the RSV promoter. This virus and the intracellular enzyme that it encodes have been characterized in detail in a separate study (see previous article; 27).

Purging Procedure. PBMCNs or CD34⁺ cells (1.8 × 10⁷) were mixed with NB-1691, SJNB-1, or SK-N-SH cells (0.2 × 10⁶) and divided into two 175-cm² flasks. To one flask, AdRSvRCE was added at an MOI of 50. The other flask was maintained as an untreated control. After 24 h, the cells in each flask were pelleted by centrifugation, the medium was decanted, and the cells were resuspended in medium containing human growth factors (300 ng/ml SCF, 10 ng/ml IL-3, and 50 ng/ml IL-6). After an additional 48 h, 5 μM CPT-11 was added to the flask of cells that had been exposed to virus. Medium was again replaced in both flasks 4 h later. Aliquots were taken from the “purged” and “unpurged” flasks and plated for clonogenic or progenitor cell assays. For clonogenic assays, a sufficient number of cells was plated such that ~3000 colonies were detected in flasks containing unpurged samples. For progenitor cell assays, six replicate wells were plated for purged and unpurged cell suspensions.

Detection by RT-PCR of Cells Expressing NB Cell Markers After Purging

Adherent and nonadherent cells were harvested from flasks containing 500,000 cells, and RNA was extracted using an RNaqueous nucleic acid extraction kit (Ambion, Inc., Austin, TX). Total cellular RNA (2 μg) was reverse transcribed using Ready-to-Go You-Prime First-Strand beads (Amer sham Pharmacia Biotech, Inc., Piscataway, NJ) according to the directions of the manufacturer. PCR analysis was done for three NB markers, TH (28), SYN, and N-MYC. Primers to detect BA were used to verify the integrity of the RNA and as a positive control for the RT-PCR reactions. Primers used for detection of the above RNAs were: TH5, GTTGTCAGAGTTCGAACTTG; TH3, GATATTGTCTTCCCCGGAAGCGCTAGA; ST5, GAGTACAAAAGGCTCTTCTTAG; SYN3, TGGCATAGTACGCGTAGTGA; NMYC5, GGGACTGGTTTCTGGCGAGAAC; NMYC3, ACTGGAGTGGTGGGTTCTG; BA5, ACTGGAGCACACACTTCTCAATAGCTGCG; and BA3, CGTICATACCTCGTGGCTGATCCATCGGC.

Annealing temperatures were: TH primers, 62°C; SYN primers, 60°C, NMYC primers, 60°C, and BA primers, 60°C. Takara Taq DNA polymerase (Panvera Corp., Madison, WI) was used to amplify cDNAs as detailed in the product brochure and with the following amplification scheme. An initial denaturation at 94°C for 5 min was followed by 1 min at 94°C, 1 min at the
appropriate annealing temperature, and 1.5 min at 72°C. The last three steps of
the program were repeated for 20 cycles, at which time additional DNA
polymerase and deoxynucleotide triphosphates were added to 8 μl of the initial
reaction mixture, and 20 more amplification cycles were carried out. RT-PCR
products were separated by agarose gel electrophoresis, and Southern analysis
was performed using 32P-labeled oligodeoxynucleotides (29). Sequences of the
probes were: GTTCGACCCTGACCTGGACT for TH; GAGCTGAGAGAC-
CCTGTGACCTCGGGA for SYN; and CTCCTGGTTTTCCCAGAAAAGC-
CAG for N-MYC.

The N-MYC primers detected mRNA encoding both the Mr 57,000 and
Mr 54,000 isoforms of this protein. Each set of primers spanned an intron to
eliminate signals that might be contributed by low levels of genomic DNA in
the RNA preparations. Probe sequences did not overlap primer sequences.

RESULTS

The long-range goal of the experiments that follow is to eradicate
the clonogenic potential of NB cells and maintain the ability of
hematopoietic stem cells to repopulate bone marrow. In vitro exper-
iments to assess the feasibility of accomplishing this goal using NB
cell lines, an adenoviral vector that encodes an intracellular form of
rabbit CE, and CPT-11 are presented.

Efficiency of Adenoviral Transduction of NB Cell Lines,
Primary NB Cells, and Human WBCs

We used a replication-deficient Ad encoding bacterial β-gal
(AdRSV β-gal) to assess the transduction efficiency of Ad for tumor
or tumor-derived cells, PBMCNs, and CD34+ cells. In the experiment
shown in Fig. 1, NB-1691 cells and primary tumor cells were exposed
to an MOI of 0 (i.e., no virus) or 50 of AdRSVβgal and then incubated
with X-gal. Transduction efficiency was dose-dependent (data not
shown), and an MOI of 50 was sufficient to transduce 100% of both
NB-1691 cells and primary NB cells. Data in Table 1 show that an
MOI of 50 is also sufficient to transduce 100% of cells of four
additional human NB cell lines. In contrast, PBMCNs were not
transduced even at a viral MOI of 500.

To determine whether a MOI of 50 would be sufficient to transduce
100% of tumor cells in mixed populations of cells, we exposed
mixtures of NB-1691 cells and PBMCNs to an adenoviral MOI of 50
and quantitated the percentage of NB-1691 cells that expressed
readily detectable levels of β-gal. The MOI of 50 was based on the
number of Ad particles/total number of NB-1691 cells + PBMCNs,
irrespective of the percentage of tumor cells present in the mixture.
Data in Table 2 show that for levels of “tumor cell contamination”
ranging from 1–25%, a MOI of 50 efficiently transduced all of the
NB-1691 NB cells in these mixtures. Therefore, we used a MOI of 50
for all of the subsequent purging experiments.

Adenoviral Toxicity of CD34+ and PBMCNs

We next examined the toxicity of Ad to hematopoietic cells using
a progenitor cell assay. CD34+ or PBMCNs were exposed to aden-
oviral MOIs ranging from 0–100, and the colony-forming ability of the
progenitor cells was assessed. Data in Fig. 2 show that exposure to Ad
had no effect on the colony-forming potential of CD34+ cells, but that
the viability of a subpopulation of PBMCNs decreased in a dose-
dependent manner at MOIs >50.

Adenoviral Toxicity to NB Cells

We also assessed the toxicity of Ad to NB cell lines. Cells were
exposed to various MOIs of Ad and plated to evaluate clonogenic

Fig. 1. Transduction efficiency of AdRSVβgal in NB-1691 and primary NB cells. NB-1691 cells or primary NB cells were exposed to an adenoviral MOI of 0 or 50 for 24 h.
Forty-eight h after removal of virus, cells were exposed to X-gal substrate overnight. The method is detailed in “Materials and Methods.”
survival. A dose-dependent relationship was seen between viral MOI and clonogenic survival for the four cell lines evaluated (Fig. 3). Toxicity varied among the cell lines, but an MOI of 50 decreased the clonogenic survival of all of the cell lines by 10–50%. We conclude that the use of an adenoviral MOI of 50 in purging protocols will likely contribute to tumor cell toxicity, independent of transgene expression or chemotherapeutic intervention.

Toxicity of CPT-11 on NB and CD34+ Cells

We next determined the concentration of CPT-11 required to reduce the clonogenic potential of NB cells essentially to zero for five NB cell lines (Table 3) and the maximum concentration of CPT-11 that had little or no effect on the colony-forming potential of PBMNCs or CD34+ cells (IC_{50-10} concentration of drug required to kill 0–10% of cells; Fig. 4 and Table 3). Cells were exposed to a range of CPT-11 concentrations for 4 h, and clonogenic or progenitor cell assays were performed. Data in Table 3 show that for the NB cell lines, the concentration of CPT-11 required to reduce the number of drug-treated colonies to a minimum of 90% of untreated controls.

Sensitizing Tumor Cells to CPT-11 Using Adenoviral Delivery of the cDNA Encoding Rabbit CE

Three NB cell lines (NB-1691, SJNB-1, and SK-N-SH) were then exposed to an adenoviral MOI of 50 for 24 h, to a range of concentrations of CPT-11 for 4 h, or to both. There was a 48-h virus-free period between exposure to Ad and exposure to drug. The amount of rCE activity detectable in lysates of aliquots of cells from each

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Table 1  Efficiency of adenoviral transduction of NB and WBCs

<table>
<thead>
<tr>
<th>Cells</th>
<th>MOI</th>
<th>Percentage of cells transduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>NB-1691</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>NB-1643</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>SJNB-1</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>IMR32</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>Primary tumor cells</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>PBMNCs</td>
<td>500</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2  Transduction efficiency of Ad for mixtures of NB-1691 NB cells with human PBMNCs

<table>
<thead>
<tr>
<th>Cell mixture</th>
<th>Percentage of NB cells transduced at MOI of 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% NB/99% PBMNC</td>
<td>100</td>
</tr>
<tr>
<td>5% NB/95% PBMNC</td>
<td>100</td>
</tr>
<tr>
<td>10% NB/90% PBMNC</td>
<td>100</td>
</tr>
<tr>
<td>25% NB/75% PBMNC</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3  Relative sensitivity of NB cells and CD34+ cells to CPT-11

<table>
<thead>
<tr>
<th>Cells</th>
<th>IC_{50-10}</th>
<th>No colonies detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+</td>
<td>5–10 μM</td>
<td>50 μM</td>
</tr>
<tr>
<td>NB-1643</td>
<td>50 μM</td>
<td>50 μM</td>
</tr>
<tr>
<td>SJNB-1</td>
<td>50 μM</td>
<td>50 μM</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>100 μM</td>
<td>100 μM</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>100 μM</td>
<td>100 μM</td>
</tr>
<tr>
<td>NB-1691</td>
<td>100 μM</td>
<td>100 μM</td>
</tr>
</tbody>
</table>
cell line at the time of exposure to CPT-11 was 4,239 ± 57, 13,235 ± 3,932, and 4,068 ± 367 for SJNB-1, SK-N-SH, and NB-1691 cells, respectively. Under similar conditions, 12.1 ± 2.1 units of CE activity are seen in PBMNCs.

Data from the clonogenic assays, Fig. 5 (top, SJNB-1 cells; middle, SK-N-SH cells; and bottom, NB-1691 cells), show that exposure to AdRSVrCE and 1–5 μM CPT-11 eliminated the clonogenic potential of each of the three NB cell lines.

**Purging NB Cells from PBMNCs or CD34+ Cells**

Taken together, results suggest that exposure of NB cell lines to AdRSVrCE (MOI, 50) for 24 h, followed by a 48-h virus-free interval and subsequent exposure of cells to 1–5 μM of CPT-11 for 4 h, should be selectively toxic to the NB cells and maintain the colony-forming potential of hematopoietic progenitor cells. However, all of the above experiments were done with either 100% NB cell lines or 100% hematopoietic cells. Realistically, samples to be purged will contain <1–10% tumor cells in a background of WBCs. Two types of experiments with mixed cell populations were done to assess the efficacy as well as the toxicity of the protocol.

**RT-PCR for NB Cell Markers in Purged Samples.** Cell suspensions (10^7 cells) containing mixtures of 90% fresh PBMNCs and 10% NB-1691 cells were “purged” by the above protocol, and 24 h after exposure to CPT-11, nonadherent and adherent cells were harvested. RNA was extracted from the cell mixtures, and RT-PCR/Southern analyses were done to assess persistence of N-MYC, SYN, and TH RNA expression after purging. Fig. 6 shows that mRNA for N-MYC, SYN, and TH is readily detectable in mixtures of 90% PBMNCs/10% NB-1691 cells that have not been exposed to AdRSVrCE and CPT-11 but is undetectable in RNA extracted from mixtures of cells exposed to virus and drug. The sensitivity of detection by this method is 1 NB-1691 cell in 10^7 PBMNCs. Full characterization of the method is reported in a separate manuscript.4 These results suggest that even in mixed cell populations, the described purging protocol is toxic to cell lines derived from primary NB tumors.

**Clonogenic and Hematopoietic Progenitor Cell Assays.** We next exposed mixtures of 10% NB cell lines (NB-1691, SJNB-1, or SK-N-SH cells) + 90% CD34+ cells to virus and CPT-11 by the above protocol, and, immediately after exposure to CPT-11, aliquots of the cell suspensions were plated for clonogenic assays or hematopoietic progenitor cell assays. Data from these assays are tabulated in Table 4 and show that the purging protocol maintained the ability of the

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4 S. M. Guichard and M. K. Danks, Detection of synaptophysin and tyrosine hydroxylase by RT-PCR predicts progression of disseminated neuroblastoma in peripheral blood and bone marrow in a preclinical model; application to human samples. Manuscript in preparation.
hematopoietic progenitor cells to form colonies in methylcellulose and eradicated the clonogenic potential of all of the three NB cell lines in these cell mixtures. No bystander effect on the CD34+ cells was observed. The data also show that each component of the protocol, i.e., virus alone and CPT-11 alone, contributed to the toxicity of the NB cells, but that neither reagent alone was sufficient to effect the death of 100% of viable tumor-derived cells. We conclude that the proposed purging method is likely effective and is selective for neuroblastoma cells and that induction of cell death is attributable to toxicity of the virus alone as well as CE overexpression and activation of CPT-11.

DISCUSSION

Numerous VDEPT approaches to purging tumor cells from hematopoietic cells have been reported (14–17), and several have used Ad as a selective delivery mechanism for this application. However, neither purging studies using the enzyme/prodrug combination rabbit CE/CPT-11 nor any VDEPT methods targeting NB have been reported previously. Other novel findings reported here include the observation that primary NB cells and NB cell lines are transduced by Ad at approximately equal MOIs. Furthermore, the method detailed here may be applicable even if a relatively high tumor burden is present, because the “purging” was effective with 100% NB-1691, SJNB-1, or SK-N-SH NB cells or with mixtures of 10% of these cell lines with 90% human CD34+ or PBMCNs. The efficacy of the described system is likely attributable to the combination of viral toxicity, overexpression of a rabbit CE, and tumor cell–specific activation of the prodrug CPT-11. The rationale for developing the above system is based in part on observations that in vivo NBs are relatively sensitive to CPT-11 (22, 23).

VDEPT methods targeting other solid tumors that contain bone marrow have been reported (14–17). Similar to results with NB cells reported in this study, breast cancer cell lines (15), a cervical carcinoma cell line (30), and primary breast cancer tumor samples (15) were also found to be >96–100% transduced at MOIs of 50–100 after a 2–24 h exposure to virus. Also, previous work (14–17) has shown that Ad transduces hematopoietic cells inefficiently, allowing for preferential delivery of cDNAs to tumor cells and, ultimately, selective tumor cell kill. On the basis of a comparison of our results of adenoviral toxicity for PBMCNs compared with CD34+ cells, it appears that a subset of hematopoietic cells is susceptible to adenoviral transduction, but that CD34+ cells are not part of this subset.

Overall, the experiments presented here suggest that Ad, CE, and CPT-11 represent a potentially useful VDEPT approach. However, similar to Hsvtk and Escherichia coli cytosine deaminase, the transgene expressed in our study is not of human origin; therefore, it is possible that overexpression of rCE will produce an immune response. Although an immune component might potentially be beneficial, a significant immune response to this protein after reinfusion of purged cells is considered unlikely for two reasons. The first is that patients who receive autologous transplants are heavily pretreated and immune-compromised; the second is that the rCE used is an intracellular protein that is 81% identical to a human CE (31).

Another consideration is that of a potential bystander effect. Unlike the activated form of ganciclovir, which requires gap junctions to diffuse from cell to cell (32), SN-38, the active form of CPT-11, diffuses freely through cell membranes (19). It seems more likely that a bystander effect would be seen with SN-38 than with ganciclovir triphosphate. However, our data show no bystander effect (Table 4) with the intracellular form of the CE used in this study. It is likely that the volume of medium in the tissue culture flasks (5–20 ml) dilutes any SN-38 that diffuses into the medium to ineffective concentrations. Therefore, it is not anticipated that a bystander effect on the CD34+ cells will be a major problem with the described method.

As indicated above, different approaches to purging have been investigated by several laboratories. In 1995, Clarke et al. (16) made the critical observation that Ad transduces hematopoietic cells inefficiently and suggested that adenoviral vectors encoding bcl-xL could be used to purge NB or breast cancer cells before autologous transplant. The report by Clarke et al. (16) also included a description of purging a mixture of 1% SHSY-5 NB cells and hematopoietic cells, but no data were shown regarding the efficacy and toxicity of these experiments. Subsequently, immunomagnetic separation has also been shown by Cheung et al. (33), using the method of Reynolds et al. (34), to be efficacious in reducing an original tumor burden of ≤1% by three to five logs. Compared with the method of Clarke et al. (16) and that of Reynolds et al. (34), the method detailed in the current study has the advantage of being efficacious when the percentage of tumor cells exceeds 1%. In the current study, experimental emphasis is placed on detection of remaining tumor cells rather than degree of depletion. RT-PCR data suggest that it may be possible to achieve essentially complete purging, irrespective of the log depletion required to achieve this goal.

In conclusion, an in vitro VDEPT approach to purging NB cells from hematopoietic cells using adenoviral delivery of the cDNA for rabbit CE and CPT-11 appears to be an effective method for eradicating NB cells, as assessed by clonogenic potential and by RT-PCR for markers of NB-derived cell lines, while maintaining the clonogenic potential of progenitor cells in populations of frozen/thawed CD34+ cells or of fresh PBMCNs. Preliminary experiments underway to assess marrow repopulation of nonobese diabetic severe combined immunodeficient mice also indicate that the described method does not affect the ability of nonobese diabetic severe combined immunodeficient repopulating cells to engraft sublethally irradiated mice.

REFERENCES


A Virus-directed Enzyme Prodrug Therapy Approach to Purging Neuroblastoma Cells from Hematopoietic Cells Using Adenovirus Encoding Rabbit Carboxylesterase and CPT-11


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