Cancer Chemother Pharmacol (2006) 58: 117–128
DOI 10.1007/s00280-005-0156-7

ORIGINAL ARTICLE

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Enforced expression of cytosolic 5′-nucleotidase I confers resistance to nucleoside analogues in vitro but systemic chemotherapy toxicity precludes in vivo selection

Received: 27 June 2005 / Accepted: 10 November 2005 / Published online: 15 December 2005

Abstract *Purpose*: Retroviral transfer of cDNA sequences that confer drug resistance can be used to protect against chemotherapy-induced hematopoietic toxicity and for the selective expansion of gene-modified cells. To successfully expand genetically engineered cells in vivo, an appropriate balance must be achieved between systemic toxicity induced by the selecting agent and the expansion of modified cells. *Method*: In this study, we investigate retroviral transfer of cytosolic 5′-nucleotidase I (cN-I) for protection and selection of gene-modified cells when treated with 2-chloro-2′-deoxyadenosine (2-CdA) and 5-fluorouracil (5-FU) alone and in combination. We also attempt to design a treatment strategy for the potential in vivo selection of cN-I-modified cells by administering 5-FU to mice prior to 2-CdA treatment. *Results*: Our results show that cN-I can be transferred by recombinant retroviruses, and that enforced expression of cN-I protects murine fibroblast and hematopoietic progenitor cells from the cytotoxic effects of 2-CdA and/or 5-FU. Furthermore, we show that the combined administration of 5-FU and 2-CdA potentiates hematopoietic stem cell toxicity. However, the treatment also results in severe myelosuppression. *Conclusion*: These results show that while cN-I provides both protective and selective benefits to gene-modified cells in vitro, selection requires a treatment strategy that is likely too toxic to consider cN-I as an in vivo selectable marker

Keywords 5′-Nucleotidase · Nucleoside analogues · Chemotherapy · Toxicity

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Introduction

The introduction of cDNA sequences that encode proteins capable of conferring resistance against cytotoxic drugs can be used to protect hematopoietic cells from the myelosuppressive effects of chemotherapy or for the in vivo selection of gene-modified cells. The ability to selectively expand gene-modified cells may be crucial for the clinical success of some gene therapy protocols that are hindered by low transduction efficiency or low level engraftment of gene-modified cells. Drug-resistance gene therapy has, therefore, been suggested as a method for selecting genetically modified cells in vivo, thus enriching the overall population of modified cells and potentially allowing for higher levels of expression of therapeutic proteins. Several cDNA sequences have been explored for drug-resistance gene therapy, which requires both the protection of genetically modified cells from cytotoxic chemotherapeutic agents as well as the ability to select these cells preferentially over unmodified cells. Vectors designed to express p-glycoprotein [22, 32], dihydrofolate reductase [2, 31], cytidine deaminase [4, 11], and alkylguanine alkyltransferase [1, 7, 35] have been studied extensively with respect to their potential as selectable markers. However, each of these

has limitations either in their capacity to select cells or in the toxicity of the selecting agent; therefore, there is a need for additional drug-resistance gene therapy systems.

Nucleotidases catalyze the conversion of nucleotide 5′-monophosphates to nucleosides by hydrolyzing the ester bond between the 5′-carbon and phosphate, and these enzymes appear to have equal efficiency when nucleoside analogue monophosphates serve as substrates [13]. In general, nucleoside analogues must be metabolized to their triphosphate forms and incorporated into DNA in order to exert cytotoxic effects. Levels of deoxycytidine kinase, which converts the nucleoside analogue to its monophosphorylated form, and nucleotidase are most accountable for the amount of active metabolite that is found in cells [9]. A relationship between nucleotidase activity and clinical response to nucleoside analogues has been shown, suggesting a role for nucleotidases in drug resistance [12, 20].

The nucleoside analogue 2-chloro-2′-deoxyadenosine (2-CdA) is used successfully in the treatment of myelodysplasias such as hairy cell leukemia and chronic lymphocytic leukemia [15, 19]. Previous studies have shown that 2-CdA enters cells through the concentrative and equilibrative nucleoside transporters and is effluxed through equilibrative nucleoside transporters [13, 24]. The 2-CdA is phosphorylated intracellularly to 2-CdAMP by deoxycytidine kinase and further metabolized to 2-CdADP by AMP kinase. 2-CdADP is phosphorylated by nucleoside diphosphate kinase to become the active metabolite 2-CdATP, which can be incorporated into replicating DNA and leads to strand breakage followed by apoptosis [16, 21, 26]. Since the levels of deoxycytidine kinase and nucleotidase are most involved in regulating the triphosphate metabolite levels in cells [9], it is assumed that nucleotidases compete with AMP kinase for 2-CdAMP binding. Presumably high nucleotidase levels can inhibit the accumulation of 2-CdAMP by catalyzing the removal of the 5-monophosphate, which allows export of nucleoside from the cell. In vitro, 2-CdA has little effect on non-stimulated $CD34^+/CD38^-$ hematopoietic progenitor cells. However, more mature progenitor cells are sensitive to the drug [10], suggesting it is the cycling nature of these cells compared to the quiescent $CD34^+/CD38^-$ cells that is responsible for the increased toxicity.

Hunsucker et al. [18] characterized human cytosolic nucleotidase-I (cN-I) and demonstrated that enforced expression of cN-I in HEK293 and Jurkat cells can increase the 2-CdA IC_{50} value by 49-fold and greater than 400-fold, respectively. We hypothesized that retroviral transfer of cN-I would provide protection to transduced cells against 2-CdA-induced cytotoxicity. In the current study, we demonstrate that cN-I can be transferred by retroviral vectors, and that transduction of murine fibroblast or hematopoietic progenitor cells with the retrovirus encoding cN-I confers resistance to 5-FU and 2-CdA, both individually and in combination. Sufficient protection is conferred to the transduced cells to allow for efficient selection of gene-modified cells. Since 2-CdA appears to be more toxic to dividing hematopoietic cells, we tested whether inducing murine hematopoietic progenitor cells to cycle using a single injection of 5-FU prior to 2-CdA treatment could selectively increase toxicity to quiescent hematopoietic cells. At doses of 5-FU and 2-CdA that are not toxic to these primitive cells, the combined treatment showed synergistic toxicity to hematopoietic progenitor and stem cells. However, the concentrations of 5-FU and 2-CdA necessary to achieve stem cell toxicity are severely myelosuppressive. Therefore, we conclude that although cN-I can be used to protect gene-modified cells against 2-CdA toxicity, cN-I is not a likely candidate to use as a selectable marker for in vivo expansion of gene modified hematopoietic stem cells.

Methods

Virus production and construction of cell lines

The cDNA sequence encoding cN-I was cloned into the HpaI and BamHI sites of the pMSCVneo vector (Clontech, Palo Alto, CA, USA). The construct was verified by restriction analysis and DNA sequencing. The MSCV-cNI plasmid and our previously characterized MSCV-GFP plasmid were transiently transfected into separate plates of 80% confluent EcoPack cells (Clontech) grown on collagen-coated plates [27]. Plates were fed with fresh medium 24 h post-transfection and virus was collected at 12 h intervals starting 36 h post-transfection and continuing until 72 h post-transfection. Viral supernatant was filtered through a 0.45 μm filter and was either directly applied to NIH3T3 murine fibroblasts or concentrated 20-fold using polymer-based centrifugation [27] and stored at −80°C before being added to cells. All cell lines, unless otherwise noted, were maintained at 37°C and 5% CO_2 in DMEM with 10% heat-inactivated fetal bovine serum, 1,000 U/ml streptomycin, 1,000 U/ml penicillin G, and 0.25 μg/ml amphotericin B (Mediatech, Herndon, VA, USA).

To generate cell lines expressing cN-I or GFP, NIH3T3 cells were plated at approximately 50% confluency, washed, and incubated with fresh viral supernatant containing either MSCV-cNI or MSCV-GFP. Supernatant was replaced every 24 h for 2 days. Transduction efficiency was 40–60% based on the percentage of GFP^+ 3T3 cells or real time PCR for cN-I. Polyclonal cN-I cell populations were used for all survival studies, and a 3T3/GFP polyclonal cell line that was 100% GFP positive was obtained by serial dilution of the transduced 3T3/GFP population.

To confirm the presence of the cN-I cDNA sequence, PCR was performed on genomic DNA isolated from MSCV-cNI as well as control MSCV-GFP transduced NIH3T3 cells using a DNA blood and tissue isolation kit (Qiagen, Valencia, CA, USA). Primers were designed to amplify a region from the psi region to the 3′-LTR

that is 871 bp for the GFP vector and 1,281 bp for the cN-I vector: psi primer 5′-CGCCTCGATCCTCCCTTTATCC-3′ and MSCV reverse primer 5′-GGGTCTTTCATTCCCCCCTTTTTCTGG-3′. PCR was performed on a Mastercycler gradient (Eppendorf, Westbury, NY, USA) under the following conditions: initial denaturing at 94°C for 4 min; 30 cycles at 1 min per step of denaturing at 94°C, annealing at 60°C, and elongating at 72°C; and a final elongation step at 72°C for 10 min. Products were run on a 1% agarose gel and stained with ethidium bromide.

In vitro survival studies

5-FU (Sigma, St. Louis, MO, USA) and 2-CdA (Calbiochem, San Diego, CA, USA) were solubilized in dH_2O, sterile-filtered, and stored at −20°C. Thymidine-depleted serum (TD-FBS) was made using methods described previously [25, 30]. Briefly, 0.15 U/ml thymidine phosphorylase (Sigma) was added to heat-inactivated FBS and incubated at 37°C for 1 h. Thymidine phosphorylase was inactivated by incubation at 65°C for 30 min. TD-FBS was filtered and added to DMEM along with antibiotics/antimycotic, generating TD-DMEM.

All cell survival studies were performed in triplicate. For continuous drug exposure studies, cells were plated at a density of 50,000 cells/well in a 12-well plate and allowed to adhere overnight. Drug-containing medium (TD-DMEM for 5-FU survival studies) was added to the cells at appropriate concentrations. For short-term exposure studies, cells were plated as above. Drug-containing medium was added to the cells at appropriate concentrations and allowed to incubate for 3 h or 30 h, at which point the drug-containing medium was removed and cells were washed twice with PBS before the addition of fresh medium. Five days after applying drug, cells were trypsinized, resuspended in DMEM, stained with Trypan blue (Mediatech) for viability, and counted using a hemacytometer. The percent of cell survival per well was determined as the number of surviving cells in each treated well relative to the total number of cells in the untreated control well.

In vitro selection of gene-modified cells

Experiments to assess if cN-I modified cells are selectively expanded in mixed cultures were performed by plating a total of 1×10^5 cells at a 50:50 ratio of 3T3/GFP to 3T3/cNI in 6-well plates and allowed to adhere overnight. Cells were counted as above on day 0 prior to drug administration to determine the exact ratio of the starting cell population. Eight μM 2-CdA was added to all remaining wells. On days 1–5, one well of cells was counted by fluorescence microscopy to determine the number of GFP^+ cells as a percentage of the total number of cells. Cells were also plated at 3T3/GFP:3T3/cNI ratios of 100:0, 80:20, 50:50, 20:80, and 0:100 and 2-CdA was added to the mixed cultures at varying concentrations and incubated for 5 days. Cell survival was determined as a percentage of the total number of cells per well relative to the total number in the 100% cN-I wells. The percentage of GFP^+ cells per well was the number of GFP^+ cells relative to the total number of cells in each well.

Determination of cN-I copy number by quantitative real-time PCR

Approximately, 1/3 of the cells from confluent 10 cm plates of 3T3/GFP and 3T3/cNI were collected from selected cell populations on day 0 and 5. DNA was isolated using a DNA tissue isolation kit (Qiagen). Primers were designed to amplify a 140 bp fragment of the 5′ portion of the cN-I cDNA: forward 5′-GGT ACCATGGAACCTGGGCAGC-3′ and reverse 5′-CG ATTTGGGTTTCTTCTTGGGCG-3′. Each reaction for real-time PCR contained 50 ng of DNA from a single cell line, SyBr Green (Applied Biosystems, Foster City, CA, USA), and forward and reverse primers. A standard curve of known copy numbers of cN-I plasmid using 50 ng of 3T3 genomic DNA was run concurrently. Real-time PCR was run on an ABI PRISM 7000 thermal cycler (Applied Biosystems) with priming at 50°C for 2 min, initial denaturation at 95°C for 10 min, and 40 cycles of denaturation at 95°C for 15 s and annealing at 58.9°C for 1 min. Data was analyzed using ABI PRISM 7000 Sequence Software.

Retroviral transduction of hematopoietic progenitor cells

Bone marrow was harvested 72 h after a single i.p. injection of 150 mg/kg 5-FU by flushing the long bones of the hind limbs into sterile-filtered PBS. Marrow was diluted in 2% acetic acid and mononuclear cells were counted using a hemacytometer. Marrow cells were plated at a density of 2×10^6 cells in one well of a 12-well plate in 2 ml DMEM + 20% FBS with IL-3 (20 ng/ml), IL-6 (50 ng/ml), and SCF (50 ng/ml; R&D Systems, Minneapolis, MN, USA). Cells were prestimulated for 48 h and counted to determinethe total number of cells. Marrow cells were divided into two equivalent aliquots, one of which was mock-transduced using supernatant from non-transfected EcoPack cells while the other was transduced twice at 24 h intervals using concentrated MSCV-cNI ecotropic virus. Twenty-four hours after the final transduction, cells were plated in triplicate in methylcellulose (M3534; StemCell Technologies, Vancouver, BC, Canada) containing increasing concentrations of 2-CdA or 5-FU + 5 μM dipyridamole. Mock-transduced cells were used as controls. Cells were plated at a density of 15,000 cells/ml. Plates were incubated for 7 days and colonies of greater than 50 cells were counted.

In vivo drug treatments

5-FU and 2-CdA were resuspended in dH_2O and sterile-filtered prior to i.p. injections. 5-FU was administered at a concentration of 100 mg/kg on day 0, and 2-CdA was administered at concentrations of 18, 27, and 40 mg/kg on days 2–6. Peripheral blood was obtained by retro-orbital venous puncture 48 h after the final administration of 2-CdA. Blood was collected using EDTA-coated capillary tubes and analyzed using a Heska CBC-Diff veterinary hematology system (Heska, Fort Collins, CO, USA). To determine the effects of 2-CdA on bone marrow progenitor cells, bone marrow was collected into sterile-filtered PBS and mononuclear cells were counted as described above. A volume equivalent to 50,000 cells from PBS treated mice was plated in 1 ml of methylcellulose in a 12-well plate. Plates were incubated at 37°C and 5% CO_2 for 7 days and colonies of greater than 50 cells were counted by visual examination.

Competitive repopulation assay

Bone marrow cells were harvested in the manner described above from untreated C57BL6/CD45.1 and treated C57BL6/CD45.2 mice. Marrow was mixed at 1:1 v/v using 1×10^6 CD45.1 cells as the control volume. The mixed marrow was transplanted via retroorbital injection into lethally irradiated (550×2 rad, 4 h apart) CD45.1 mice. Peripheral blood was collected from transplant recipients and hematopoietic repopulation was analyzed at 1, 3, 4, and 6 months following transplant. After red cell lysis, the collected cells were stained with CD45.1-FITC and CD45.2-PE (Pharmingen, San Jose, CA, USA) to quantify CD45 positive hematopoietic cells arising from either the untreated or drug treated donor.

Results

The purpose of this study is to determine whether gene transfer of cN-I can be used to protect and select gene-modified cells and if so, whether a treatment schedule can be devised that will allow for the in vivo selection of cN-I-modified cells. The proposed mechanism by which cN-I prevents the toxic metabolite 2-CdATP from accumulating in the cell is shown in Fig. 1. We hypothesize that enforced expression of cN-I from recombinant retroviral vectors will prevent phosphorylation of the nucleoside analogue thereby limiting accumulation of the toxic metabolite 2-CdATP.

Retroviral transfer of cN-I can protect cells against 2-CdA-mediated toxicity

In order to test whether cN-I can confer resistance to cells through retroviral transfer, we constructed an MSCV-cNI vector, produced ecotropic recombinant retrovirus, and transduced NIH3T3 cells. An MSCV-GFP construct

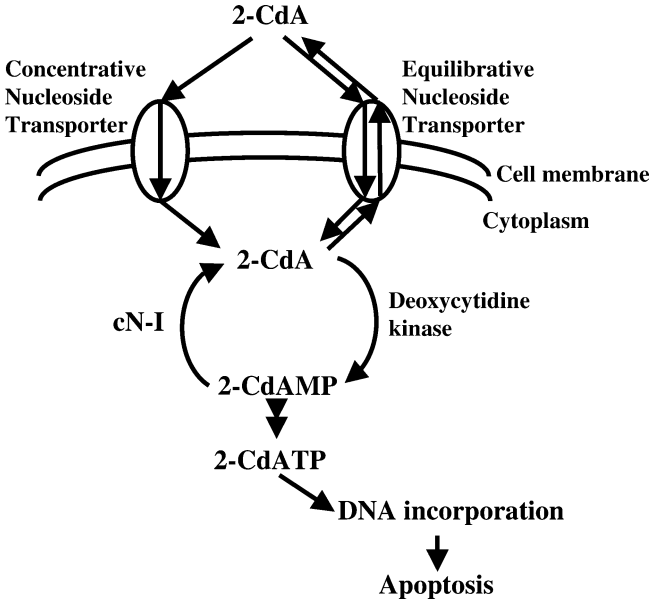

Fig. 1 Proposed mechanism of cN-I protection against 2-CdA toxicity. Both concentrative and equilibrative nucleoside transporters are used to import 2-CdA into cells. Intracellularly, 2-CdA is metabolized to 2-CdAMP by the rate-limiting enzyme deoxycytidine kinase. cN-I is presumed to compete with AMP kinase for binding to 2-CdAMP. Overexpression of cN-I can prevent the further metabolism of 2-CdAMP to the toxic metabolite 2-CdATP by catalytic conversion back to its dephosphorylated state. Equilibrative nucleoside transporters can efflux 2-CdA from cells to prevent cellular toxicity

was used as a control for all in vitro experiments. The presence of the full-length cN-I transgene was confirmed by PCR analysis of genomic DNA isolated from the 3T3/cN-I cell population (inset to Fig. 2a). Real-time PCR performed on the 3T3/cN-I population revealed an average of 0.5 copies of cN-I per cell (data not shown). Survival studies were performed using 3T3/GFP and 3T3/cNI cells in which they were treated with increasing concentrations of drugs for either continuous or short-term exposure. Continuous exposure to 2-CdA under normal culture conditions showed that cN-I expression confers approximately 11-fold resistance to 3T3 cells (Fig. 2a). Short-term exposure to higher concentrations of 2-CdA also produced an increase in resistance, although not as large as when cells were continuously exposed to lower drug concentrations (Fig. 2b).

In addition to 2-CdA, cN-I can also inactivate other cytotoxic nucleoside analogues that require phosphorylation to become cytotoxic. 5-FU is metabolized intracellularly to several toxic metabolites that can incorporate into RNA and disrupt strand synthesis or inhibit thymidylate synthase and disrupt DNA synthesis [23]. We tested if cN-I transduced cells were also resistant to 5-FU by plating cells in medium containing thymidine-depleted FBS and various concentrations of 5-FU. As shown in Fig. 2c, 3T3/cN-I cells are over seven-fold more resistant to 5-FU compared to 3T3/GFP cells. To determine if cN-I confers resistance to combinations of 5-FU and 2-CdA, cells were treated

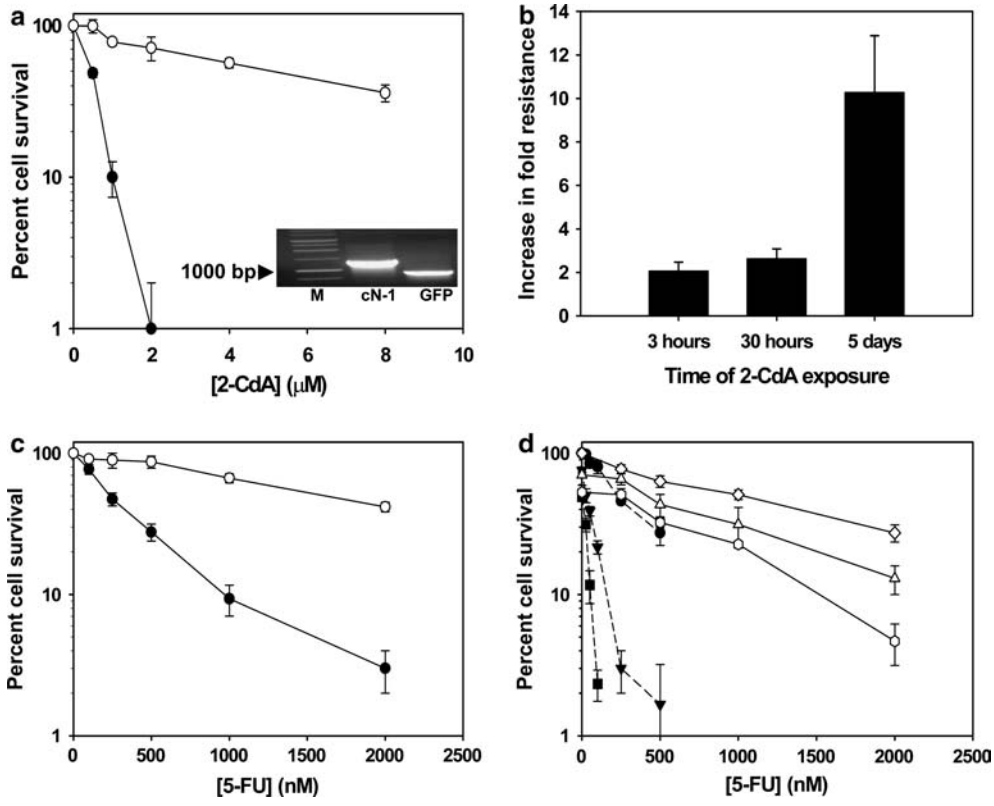

Fig. 2 cN-I confers resistance to gene-modified cells. NIH 3T3 cells were transduced with retroviruses encoding either cN-I or GFP. Transduced cells were challenged with various concentrations of 2-CdA, 5-FU or a combination of both. The percentage of surviving cells after drug treatments were determined relative to the number of surviving cells in untreated control wells. All points are the mean ± SD. **a** 2-CdA was added on day 1 and cells were continuously exposed to drug for 5 days. Inset: PCR of genomic DNA from 3T3/cN-I cells (1,281 bp) or 3T3/GFP cells (871 bp) with primers that amplify the entire cN-I and GFP cDNA sequence. **b** Comparison of fold increases in 2-CdA resistance for cN-I cells compared to GFP cells after treatment with 2-CdA for 3, 30 h, or 5 days. **c** Continuous treatment of cells with various doses of 5-FU in TD-DMEM medium. **d** Continuous treatment of cells in both 5-FU and 2-CdA. *Open symbols* represent cN-I tranduced cells in media containing 5-FU (concentrations of which are shown within the figure) and 0 nM 2-CdA (*diamond*), 1,000 nM 2-CdA (*triangle*), or 2,000 nM 2-CdA (*circle*). *Solid symbols* represent GFP-modified cells in media containing 5-FU with 0 nM 2-CdA (*filled circle*), 250 nM 2-CdA (*filled triangle down*), or 500 nM 2-CdA (*filled square*)

with continuous exposure to both drugs. As shown in Fig. 2d, the 5-FU IC_{50} value for 3T3/GFP cells dramatically decreases with increasing concentrations of 2-CdA. In contrast, cN-I transduced cells survive the combined drug challenge. GFP cells plated in 250 nM 2-CdA have a 5-FU IC_{50} value of 27 nM, a decrease of 8.5-fold over GFP cells treated with 5-FU alone. In contrast, cN-I cells plated in eightfold more 2-CdA (2 μM) have a 5-FU IC_{50} value of 420 nM, a decrease of only fourfold compared to cN-I cells plated in 5-FU alone. cN-I, therefore, (1) can be transferred by recombinant retroviruses, (2) confers resistance to 2-CdA, (3) confers resistance to 5-FU, and (4) confers resistance to combinations of 2-CdA and 5-FU.

cN-I-transduced cells can be preferentially selected in vitro

Effective drug resistance gene therapy requires that the gene-modified cells survive preferentially over unmodified cells when treated with the drug to which the gene confers resistance. Figure 3a shows that 3T3/cN-I cells can be preferentially expanded over 3T3/GFP cells over the course of 5 days. Cells were initially plated at a 50:50 ratio and 2-CdA was added. Cells were counted each day to determine the number of GFP^+ cells relative to the total number of surviving cells. The ratio of GFP^+ cells decreased from 50% of the initial cell population to approximately 6% after 5 days of 2-CdA treatment. Real-time PCR was performed on DNA isolated on days 0 and 5 and showed there was a statistically significant increase in cN-I copy number on day 5 compared to day 0, from approximately 0.25 to 1 copy/cell. These results indicate that cN-I-expressing cells are enriched while GFP^+ cells are selectively eliminated.

To further show the protective effects of cN-I against 2-CdA toxicity, 3T3/GFP, and 3T3/cN-I cells were plated at different ratios and treated with 4, 8, or 16 μM 2-CdA. After 5 days of selection, cells were analyzed for both survival and GFP expression. As shown in Fig. 3b,

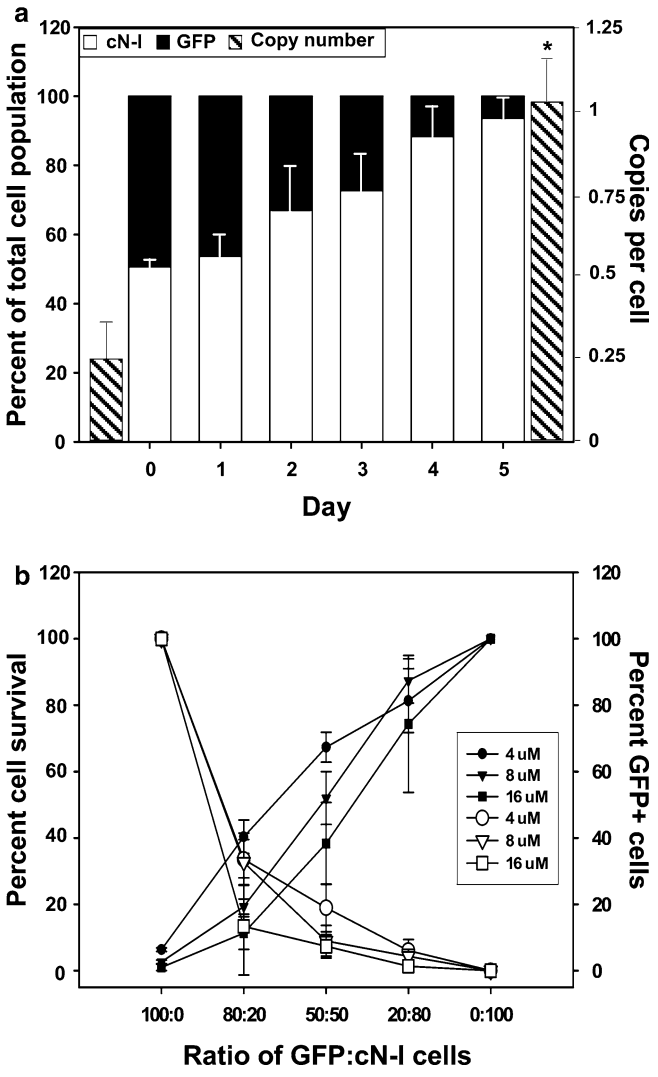

Fig. 3 In vitro selection of cN-I-modified cells. Mixed cell cultures were established with varying ratios of cNI (*open bars*) and GFP (*closed bars*) expressing cells and incubated with 2-CdA. Genomic DNA was isolated from the mixed cultures and used to assess the cN-I proviral copy number by real time PCR on days 0 and 5. **a** cN-I and GFP-expressing cells were plated at a 50:50 ratio and treated with 8 μM 2-CdA. Cells were counted daily to determine changes in GFP expression compared to the original day 0 ratio. *Hatched bars* on days 0 and 5 show the significant increase in cN-I gene copy number per cell (Student's *t*-test, $p < 0.05$). **b** cNI and GFP-expressing cells were plated at the ratios indicated within the figure and incubated for 5 days with varying concentrations of 2-CdA. The surviving cells were counted to determine both survival and the change in the percentage of GFP-expressing cells compared to the starting ratio. *Open symbols* represent the percentage of GFP^+ cells and *closed symbols* represent the percentage of surviving cells

there is a 2-CdA dose-dependent decrease in GFP^+ cell survival. Virtually no GFP^+ cells survive challenges of 8 and 16 μM 2-CdA, indicating these doses are lethal to non-cN-I cells after 5 days of treatment. However, cell survival clearly increases as the percentage of cN-1+ cells increases.

Transduction of cN-I protects murine hematopoietic progenitor cells from 2-CdA or 5-FU toxicity

Based on our results demonstrating that cN-I protects fibroblast cells, we next studied whether cN-I can protect murine progenitor cells. Bone marrow was harvested from 5-FU-treated mice and prestimulated with IL-3, IL-6, and SCF for 48 h. Half the marrow was transduced with concentrated cN-I/ecotropic virus and half was mock-transduced. After transduction, cells were plated in methylcellulose containing 2-CdA or 5-FU + 5 μM dipyridamole. Approximately 40% of marrow cells transduced with cN-I survived treatment of 200 nM 2-CdA, a concentration that eliminated >90% mock-transduced cells, demonstrating the ability of cN-I to protect murine progenitor cells from the toxic effects of 2-CdA (Fig. 4). In addition, over 60% of cN-I transduced cells survived drug treatment with 600 nM 5-FU + dipyridamole, which was toxic to approximately 90% of mock-transduced bone marrow progenitors. These results demonstrate the ability of cN-I to protect murine progenitor cells from the toxic effects of 2-CdA and 5-FU.

Hematopoietic progenitor cells are insensitive to 2-CdA in vivo

With a goal of determining if 2-CdA can be used as a selecting agent for hematopoietic stem cells, in vivo studies were initiated to establish the hematopoietic

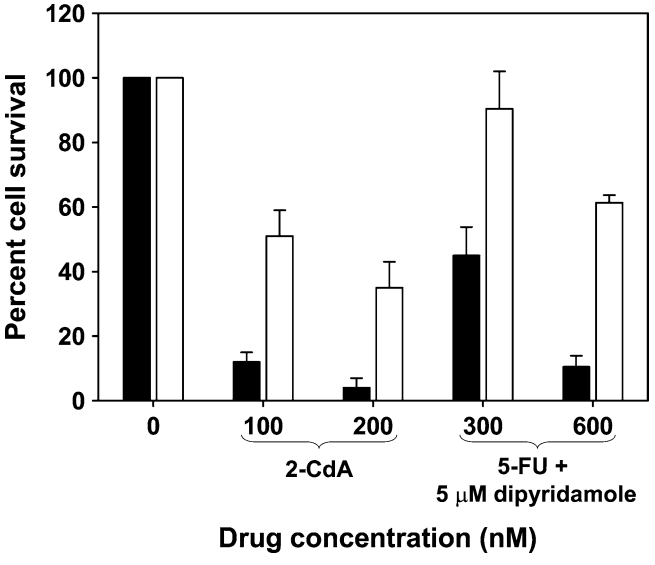

Fig. 4 Protection of transduced bone marrow progenitor cells from the toxic effects of 2-CdA or 5-FU + dipyridamole. cN-I-transduced and mock-transduced marrow was plated in semisolid methylcellulose with increasing doses of 2-CdA or 5-FU + 5 μM dipyridamole, as labeled within the figure. Colonies derived from drug-treated hematopoietic progenitor cells were determined through day 7 ($n = 3$)

toxicity profile of 2-CdA toward hematopoietic stem and progenitor cells in mice. Previous studies have shown that 2-CdA is toxic to lineage committed murine hematopoietic and CFU-S cells in vivo but human $CD34^{+}/CD38^{-}$ cells are insensitive to 2-CdA in vitro [10, 14]. Similar to previous studies, we show that 2-CdA decreases the circulating number of white blood cells, granulocytes and lymphocytes and a transient decrease in body weight occurs in 2-CdA treated mice compare to control mice (Fig. 5 a–d). Interestingly, similar to studies administering myelosuppressive doses of antifolates [3], the number of myeloid progenitor cells per femur increased after 2-CdA treatment even though the number of bone marrow mononuclear cells decreased, indicating hematopoietic progenitor cells in vivo are insensitive to 2-CdA (Fig. 6 a–d).

Pretreatment with 5-FU sensitizes hematopoietic cells to 2-CdA

Because the cytotoxic activity of 2-CdA appears to be more pronounced in committed hematopoietic cells, it was hypothesized that induction of hematopoietic progenitor and stem cell cycling would increase the sensitivity of these cells to 2-CdA. Previous studies have shown that 5-FU induces primitive hematopoietic stem cells to divide and that within 2–3 days of treatment, nearly 30% of stem cells have entered the cell cycle [17, 29]. We showed that cN-I confers resistance to both 5-FU and 2-CdA. Therefore, it would be expected that cN-I-modified hematopoietic cells would be protected from both drugs used in a 5-FU/2-CdA treatment regimen.

To determine if hematopoietic stem cells are sensitized to 2-CdA following a dose of 5-FU, C57BL6/CD45.2 mice were administered PBS, 5-FU alone (100 mg/kg), 2-CdA alone (40 mg/kg), or 5-FU (100 mg/kg) followed by 2-CdA at doses of 18, 27, or 40 mg/kg (groups F18C, F27C, and F40C, respectively) via i.p. injection. 5-FU was administered 48 h prior to the start of the 2-CdA treatment. 2-CdA was administered at 24 h intervals for 5 days. Mice were weighed daily and peripheral blood was taken 48 h after the final injection of 2-CdA. Animals were then sacrificed, and bone marrow was collected from the long bones of the

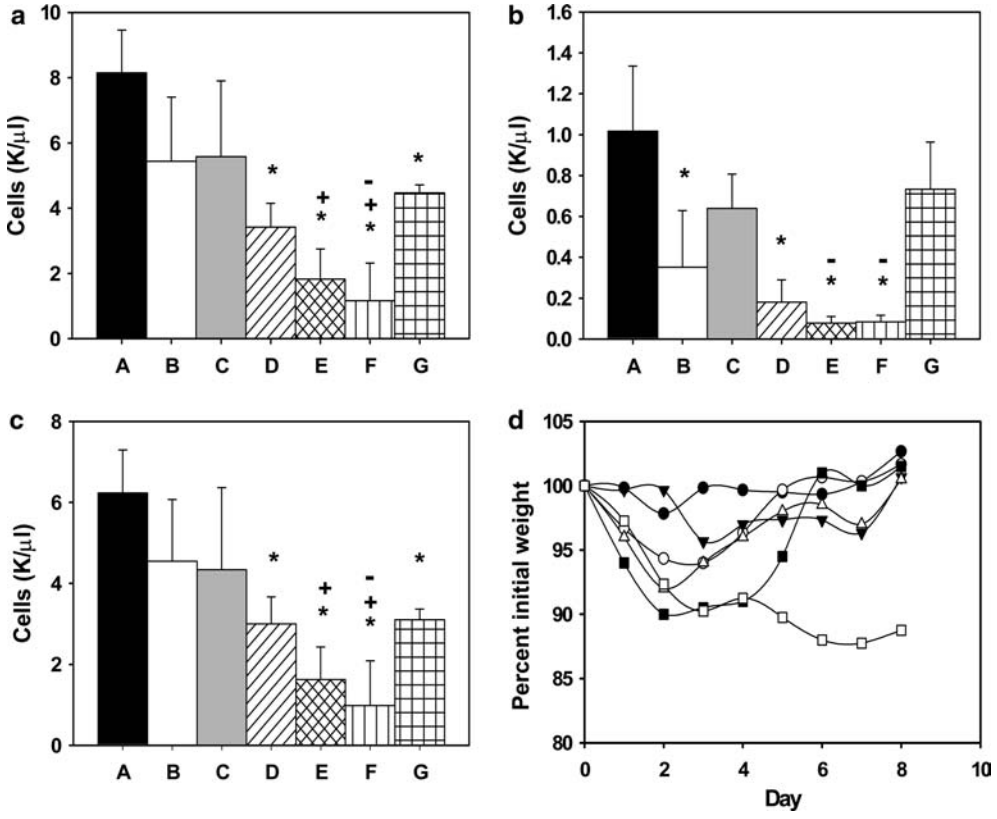

Fig. 5 Effects of 2-CdA on Hematopoietic parameters and body weight. Mice from all groups were bled 48 h after the final 2-CdA treatment and analyzed for complete blood counts. *Panels A*, *B*, and *C* show the white blood counts, granulocyte counts and lymphocyte counts, respectively. Within *panels A–C*, *bar A* represents mice treated with PBS; *bar B* represents 5-FU (100 mg/kg), *bar C* represents 40 mg/kg 2-CdA, *bar D* represents 5-FU +18 mg/kg 2-CdA, *bar E* represents 5-FU +27 mg/kg 2-CdA, *bar F* represents 5-FU +40 mg/kg 2-CdA, and *bar G* represents 40 mg/kg 2-CdA followed by 5-FU. All data plotted is the mean ± SD. * comparison to PBS. + comparison to 5-FU. - comparison to 2-CdA. *Panel D* shows mouse weights over the course of treatment: PBS *(filled circle)*, 5-FU (*circle*), 2-CdA (*filled triangle down*), 5-FU +18 mg/kg 2-CdA (*triangle*), 5-FU +27 mg/kg 2-CdA (*filled square*), and 5-FU +40 mg/kg 2-CdA (*square*)

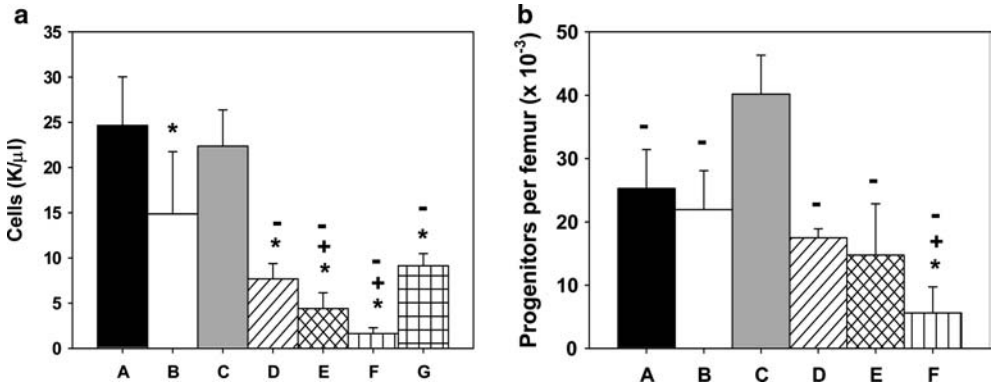

Fig. 6 Effect of 5-FU and 2-CdA treatment on bone marrow mononuclear and progenitor cells. The number of mononuclear cells from bone marrow is shown in *panel A* and the number of progenitor cells per femur is shown in *panel B*. The individual treatment groups are identical to the groups presented in Fig. 5. All data plotted is the mean ± SD. * comparison to PBS. + comparison to 5-FU. - comparison to 2-CdA

hind limbs and plated in methylcellulose or used in competitive repopulation assays.

All mice survived the full schedule of drug treatments. Administration of 5-FU alone causes a rapid decrease in weight on the day following the 5-FU treatment, with recovery to normal weight levels within several days (Fig. 5d). Mice treated with 2-CdA alone show a weight decrease during the 2-CdA treatment; however, their weights rapidly return to normal following treatment cessation. Both the F18C and F27C groups show a rapid decrease in weight following 5-FU injection and tend to remain low during the initial 2-CdA treatment, but returned to normal. In contrast, the weight of the F40C group rapidly decreased following 5-FU administration and continued to decrease through the administration of 2-CdA.

Following the full course of treatment, complete blood counts were analyzed from peripheral blood. All combination treatment groups had reduced numbers of white blood cells, granulocytes, and lymphocytes, compared to the PBS and 5-FU control groups (Fig. 5 a–c). In a reverse experiment, mice were first administered 40 mg/kg 2-CdA for 5 days then treated with 5-FU 48 h later. These mice had hematopoietic toxicity profiles similar to the 2-CdA only cohort. However, there was a decrease in the number of bone marrow mononuclear cells to levels below that of the 5-FU alone group, but only to that of the 5-FU followed by 18 mg/kg 2-CdA group; it was not significantly different than that of 5-FU alone (Fig. 6a).

5-FU potentiates the toxicity of 2-CdA to bone marrow mononuclear and progenitor cells

The decrease in committed blood cells in the combination treatment groups indicates that the 5-FU followed by 2-CdA treatment is more toxic than 2-CdA or 5-FU alone. To determine the effects of combining 5-FU and 2-CdA on bone marrow progenitor and stem cells, bone marrow was harvested from the hind limbs of treated mice. Mononuclear cell counts for the 5-FU alone and all combination treatment groups were significantly lower than the PBS group (5-FU, $p = 0.002$; F18C, F27C, F40C $p < 0.001$) (Fig. 6a), and each of the combination treatment groups had reduced counts compared to the 2-CdA treated group ($p < 0.001$ for all groups), but only F27C and F40C had lower numbers of mononuclear cells than the 5-FU alone group (F27C $p = 0.007$; F40C $p < 0.001$).

In order to determine the toxicity of the combination treatment to hematopoietic progenitor cells, equal volumes of bone marrow from each group were plated in methylcellulose (a volume equivalent to 50,000 cells from the PBS group). Colonies with 50+ cells were counted and used to calculate the number of progenitors per femur in each mouse. As stated above, there is an increase in progenitor cells in the cohort treated with 2-CdA alone (Fig. 6b). Only the F40C cohort had significantly fewer progenitor cells compared to both the PBS and 5-FU alone groups (both $p < 0.001$).

Combined 5-FU/2-CdA treatment is toxic to long-term repopulating hematopoietic cells

To determine if the 5-FU/2-CdA combination is toxic to hematopoietic stem cells, a competitive repopulation assay was performed in which marrow from treated CD45.2 mice was mixed with marrow from untreated CD45.1 mice, and the combined marrow was transplanted into lethally irradiated CD45.1 mice. Hematopoietic reconstitution of the transplanted mice was followed for 6 months. Peripheral blood was obtained and stained with CD45.1-FITC and CD45.2-PE, then subjected to flow cytometric analysis to determine the percentage of repopulation with CD45.1 vs. CD45.2 hematopoietic stem cells. Figure 7 shows representative data from each mouse 6 months post-transplant. With the exception of the F40C cohort, it is clear that there is

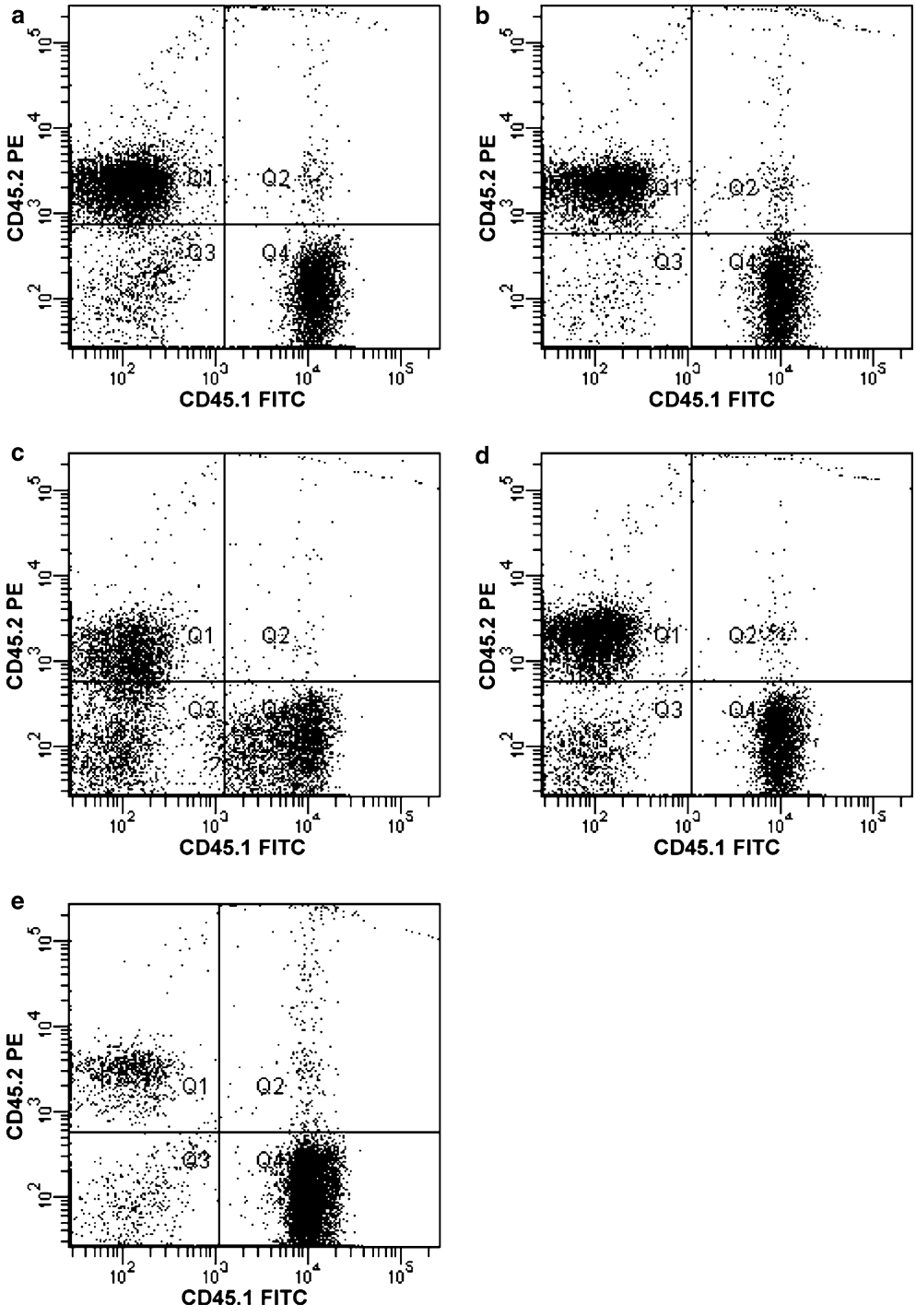

Fig. 7 Representative flow cytometric analysis of peripheral blood from competitive repopulation assays 6 months post-transplant. Mice were bled via retroorbital puncture and blood was stained with CD45.1-FITC and CD45.2-PE to determine the repopulation potential of marrow harvested from treated or non-treated mice. *Data* represent individual mice 6 months post-transplant groups receiving: **a** PBS, **b** 5-FU (100 mg/kg), **c** 5-FU (100 mg/kg) +18 mg/kg 2-CdA, **d** 5-FU (100 mg/kg) +27 mg/kg 2-CdA, **e** 5-FU (100 mg/kg) +40 mg/kg 2-CdA

virtually no difference in repopulation among the experimental groups. However, repopulation by CD45.2 marrow was dramatically decreased in the F40C cohort. The data summarized in Table 1 shows that 1 and 3 months after transplant there appears to be a decrease in repopulating cells in both the F27C and F40C groups. However, after 4 months there was essentially equal repopulation between CD45.1 and CD45.2 bone marrow in the F27C group, while the F40C group remained suppressed. These results show that long-term repopulating cells were only affected in the F40C group.

Discussion

Detailed understanding of the molecular events leading to drug resistance has allowed for the development of retroviral vectors encoding drug-resistance cDNA sequences that can be used to protect individuals from systemic hematological toxicities associated with chemotherapeutic drugs. In addition, cDNA sequences that confer drug resistance can be used for in vivo selection of gene-modified cells, thus circumventing low

Table 1 Summary of competitive repopulation assays

	Month 1	Month 3	Month 4	Month 6
PBS	41.8 ± 5.4	38.9 ± 7.7	56.1 ± 13.7	53.3 ± 8.5
5-FU	57.3 ± 5.0	41.0 ± 3.4	51.1 ± 12.6	61.1 ± 2.6
5-FU/18 2-CdA	44.0 ± 2.6	32.4 ± 3.7	40.5 ± 12.2	46.4 ± 19.2
5-FU/27 2-CdA	29.0 ± 10.8	21.7 ± 8.7	49.3 ± 17.8	41.9 ± 20.3
5-FU/40 2-CdA	11.7 ± 1.5	9.3 ± 1.3	7.8 ± 2.2	7.2 ± 1.6

Mice were bled at months 1, 3, 4, and 6 post-transplant and blood was stained with CD45.1-FITC and CD45.2-PE to differentiate between repopulation with marrow harvested from untreated or treated donor mice ($n = 3$–6)

transduction efficiencies observed in hematopoietic gene therapy studies. Effective drug resistant gene therapy strategies must meet multiple requirements, such as (1) high level resistance is conferred to gene-modified cells, (2) resistance is conferred at low copy numbers/modified cell, (3) the cDNA sequence is efficiently transferred using recombinant retroviruses, (4) the differential of drug sensitivity between modified and non-modified cells is sufficient to allow for in vivo selection of gene-modified cells, and (5) selection is achieved at drug doses that minimize systemic toxicity. To date, no selection system meets each requirement. However, gene transfer of drug resistant variants of alkylguanine alkyltransferase have been successfully used to protect hematopoietic cells against nitrosourea-induced myelosuppression as well as for in vivo selection of gene modified cells [1, 7, 35]. Other systems, such as the use of dihydrofolate reductase, confer high level resistance against selected chemotherapy agents, but in vivo selection is compromised due to systemic drug toxicity [5, 8, 28, 33, 34]. Therefore, development of successful drug resistant gene therapy strategies should involve a balance between the protective effects of transgene expression and systemic toxicity associated with the drug. In this study, we tested the hypothesis that retroviral transfer of cN-I can protect cells from the cytotoxic effects of 2-CdA and 5-FU, and that pretreatment with 5-FU to induce stem cell cycling would enhance the stem cell toxicity of 2-CdA.

It was established by Hunsucker et al. [18] that cN-I overexpression in HEK293 and Jurkat cells confers resistance to 2-CdA as well as other purine analogues. We hypothesized that overexpression of cN-I in hematopoietic stem cells could be a potential strategy for protecting and selecting gene-modified cells, especially since endogenous cN-I levels are low in primitive bone marrow cells. In the current study, we demonstrate high level protection in vitro from the cytotoxic effects induced by 2-CdA and 5-FU through retroviral transfer and expression of cN-I, for both murine fibroblast and hematopoietic progenitor cells. Our results further demonstrate that while cN-I cells are protected against low, continuous doses of 2-CdA, higher drug doses used over shorter exposure times diminishes the degree of protection. The level of protection against drug toxicity conferred by cN-I suggests it is a viable option for selection of cN-I modified cells. This was confirmed by the observation that cN-I expressing cells can be selected over GFP transduced NIH 3T3 cells following 2-CdA treatment, and there is a concomitant increase in the cN-I gene copy number following drug treatment. This data not only emphasizes the protective effect of cN-I against the toxicity associated with 2-CdA treatment, but also shows that cN-I could potentially be utilized for ex vivo selection of gene modified cells, or in vivo protection against 2-CdA induced myelosuppression.

Based on our in vitro data, it was reasonable to test whether cN-I can be used as an in vivo selectable marker. However, our initial studies showed that 2-CdA is not toxic to murine hematopoietic progenitor cells following systemic treatment. For the successful application of cN-I for in vivo selection it was, therefore, necessary to establish a selection regimen that would extend the toxicity of 2-CdA to primitive hematopoietic cell populations. It is well established that pretreatment of mice with 5-FU can be used as a method for inducing cell cycling in primitive cells [6, 17, 29]. We hypothesized that hematopoietic stem cells would be more sensitive to the effects of 2-CdA if they were first induced to cycle. As predicted, potentiation of 2-CdA toxicity to peripheral blood, bone marrow, and hematopoietic progenitor cells occurs when mice are prestimulated with a single dose of 5-FU prior to 2-CdA treatment. Interestingly, there was a significant increase in the number of hematopoietic progenitor cells in the 2-CdA only treatment group. A potential explanation for this observation is that the toxicity to committed cells results in proliferation of bone marrow progenitor cells as a method of repopulating the depleted peripheral blood cell compartments. There are several possibilities for this expansion. One prospect is that there is an as-yet-unknown mechanism by which the progenitor cell population can effectively overcome the toxicity induced by 2-CdA treatment, such as regulating nucleoside transporter activity. A second explanation deals with the time schedule of the treatment, such that the 2-CdA-induced toxicity to committed cells occurs after several treatments and that progenitor cycling is therefore not induced immediately upon the initiation of treatment. If cycling of progenitor cells takes several days to occur, there could be too few remaining 2-CdA treatments to induce toxicity in this population. This is less likely because it would be expected that treatment with 5-FU after 2-CdA would lead to increased hematopoietic toxicity, which did not occur.

Competitive repopulation assays can be used to assess the toxicity of chemotherapeutic agents to long-term repopulating cells. Using this assay the toxicity of the combination treatment of 5-FU and 2-CdA to progenitor cells does not necessarily correlate with toxicity to long-term repopulating cells. Our results demonstrate that stem cells can be targeted when 5-FU is administered prior to 2-CdA, indicating 5-FU and 2-CdA can be used to effectively eliminate primitive hematopoietic cells and potentially be a useful method for the in vivo selection of

gene-modified cells. However, combination treatments using low doses of 2-CdA are extremely myelosuppressive but not toxic to hematopoietic stem cells. Therefore, considering the myelosuppression associated with the 5-FU/2-CdA treatment schedule, we conclude it is unlikely to be a viable method for selecting cN-I-expressing hematopoietic cells. In order to consider using cN-I as an in vivo selectable marker, the development of novel drugs that could exert high level selective toxicity to primitive hematopoietic cells and to which cN-I confers resistance may be required. However, a potential utility for cN-I exists if used in combination with 5-FU only. It has been well established that two doses of 5-FU administered in close succession are stem-cell toxic [3, 17]. We are currently examining the potential of using a treatment schedule involving only 5-FU to obtain in vivo stem cell selection of gene-modified cells.

Our results demonstrate that retrovirus mediated enforced expression of cN-I confers resistance to 2-CdA and 5-FU, both individually and in combination. Although resistance is conferred to gene-modified cells at relatively low copy numbers, doses of 5-FU and 2-CdA required for killing hematopoietic stem cells is severely toxic systemically, indicating 2-CdA coupled with cN-I expression is likely not a valid system for use as an in vivo selectable marker for hematopoietic stem cells. However, we show that cN-I can be used to protect gene-modified cells against 2-CdA toxicity. Because the dose limiting side effect of 2-CdA treatment is myelosuppression, the protective properties of cN-I can be useful in drug resistant treatment strategies for the purpose of suppressing chemotherapy-induced myelosuppression. We are currently testing the benefit of using 2-CdA-resistant immunocompetent cells in the treatment of certain types of murine leukemia in the context of combining immunotherapy and chemotherapy.

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