Targeting Tumor-Initiating Cancer Cells with dCD133KDEL Shows Impressive Tumor Reductions in a Xenotransplant Model of Human Head and Neck Cancer

Nate N. Waldron¹, Dan S. Kaufman², Seunguk Oh³, Zintis Inde³, Melinda K. Hexum², John R. Ohlfest⁴, and Daniel A. Vallera³

Abstract

A novel anticancer agent was constructed by fusing a gene encoding the scFv that targets both glycosylated and unglycosylated forms of CD133 to a gene fragment encoding deimmunized PE38KDEL. The resulting fusion protein, dCD133KDEL, was studied to determine its ability to bind and kill tumor-initiating cells in vitro and in vivo. The anti-CD133 scFv selectively bound HEK293 cells transfected with the CD133 receptor gene. Time course viability studies showed that dCD133KDEL selectively inhibited NA-SCC and UMSCC-11B, 2 head and neck squamous cell carcinomas that contain a CD133 expressing subpopulation. Importantly, the drug did not inhibit the viability of hematopoietic lineages measured by long-term culture-initiating cell and colony-forming assays from sorted human CD34⁺ progenitor cells. In addition to in vitro studies, in vivo tumor initiation experiments confirmed that CD133-sorted cells implanted into the flanks of nude mice grew faster and larger than unsorted cells. In contrast, cells that were pretreated with dCD133KDEL before implantation showed the slowest and lowest incidence of tumors. Furthermore, UMSCC-11B-luc tumors treated with multiple intratumoral injections of dCD133KDEL showed marked growth inhibition, leading to complete degradation of the tumors that was not observed with an irrelevant control-targeted toxin. Experiments in immunocompetent mice showed that toxin deimmunization resulted in a 90% reduction in circulating antitoxin levels. These studies show that dCD133KDEL is a novel anticancer agent effective at inhibiting cell proliferation, tumor initiation, and eliminating established tumors by targeting the CD133 subpopulation. This agent shows significant promise for potential development as a clinically useful therapy. Mol Cancer Ther; 10(10); 1829–38. ©2011 AACR.

Introduction

Evidence has mounted over the last decade that cancers contain a small subset of their own stem-like cells termed cancer stem cells (CSC). These CSC are known to enhance tumor initiation, self-renew, and also differentiate into phenotypically diverse cancer cells with more limited proliferative potential (1–3). A point of controversy regarding CSC is the existence of plasticity between stem cells and their more differentiated derivatives, and some believe that more differentiated cancer cells can become reprogrammed and revert back to CSC (4). CSC are more resistant to current chemotherapy agents and have distinct cell surface markers (5, 6). Because of this, it is important to develop new therapeutics to specifically target CSC. CD133 (prominin-1) is a 5-transmembrane glycoprotein whose function remains unknown. It is expressed on hematopoietic, endothelial, and neuronal stem cells (7–9) and has recently been identified as a CSC marker in a variety of carcinomas as well (6, 10–13).

Targeted toxins are a class of biological drug consisting of a catalytic protein toxin chemically or genetically linked to a ligand recognizing a specific marker expressed on cancer cells (14). The catalytic destruction of the target cell is dependent on the internalization of the target receptor. A recent study has shown that antibodies targeting the CD133 receptor are efficiently internalized (15). Targeted toxins are a class of biological drug consisting of a catalytic protein toxin chemically or genetically linked to a ligand recognizing a specific marker expressed on cancer cells (14). The catalytic destruction of the target cell is dependent on the internalization of the target receptor. A recent study has shown that antibodies targeting the CD133 receptor are efficiently internalized (15). Other studies have shown that CD133⁺ cells possess a very strong ability to initiate tumors, but CD133⁻ cells do not (16, 17). Thus, we reasoned that a targeted toxin directed against CSC via CD133 might prove highly disruptive to the dynamic process of tumor initiation and progression.

A novel targeted toxin called deimmunized CD133KDEL (dCD133KDEL) was synthesized using an anti-CD133 scFv reactive against loop 2 of the extracellular domain of CD133. This scFv was taken from the monoclonal
antibody that has been shown to recognize both the glycosylated and unglycosylated forms of CD133 (18). This scFv was then cloned onto the same molecule containing a truncated form of pseudomonas exotoxin A (PE38) that has been successfully established as a clinically useful toxin (19). Studies show that fewer than 1,000 molecules of PE38 delivered to the cytosol are sufficient to bring about cell death (20). In addition, we added a Lys-Asp-Glu-Leu (KDEL) C-terminus signal to our drug that provides enhanced tumor killing by preventing luminal ER protein secretion (21). Also, genetic engineering was used to address a major shortcoming of targeted toxins, their immunogenicity. To accomplish this, the PE toxin was mutated to remove immunogenic epitopes that were recently mapped (22). Studies show that 3 separate targeted toxins made with this deimmunized variant have highly reduced antitoxin levels in mice despite multiple treatments with the drug compared with the nonmutated parental form (23–25).

In this article, we show that dCD133KDEL specifically kills CD133+ tumor-initiating cells and can arrest the proliferation of head and neck carcinoma cells in vitro and in vivo. dCD133KDEL is a novel deimmunized targeted toxin that target tumor-initiating cells and is effective at preventing tumorigenesis and treating established tumors. This represents a significant and highly selective tool that can be used to study stem cell populations and as a possible clinical adjunct to chemotherapy.

Materials and Methods

Construction of dCD133KDEL
dCD133KDEL was synthesized from the fusion of DNA fragments encoding the scFv portion (anti-CD133scFv) of clone 7 (18) and a deimmunized, truncated form of pseudomonas exotoxin 38 (23–25). The assembled fusion gene contained (5′–3′) an NcoI restriction site, the ATG initiation codon, the gene for CD133 scFv, the DNA sequence encoding a 7 amino-acid EASGGPE linker, the gene encoding for the first 362 amino acids of truncated deimmunized with the DNA sequence for KDEL replacing the REDLK at the C-terminus, followed by a NolI restriction site at the 3′ end of the fusion gene. The resulting 1,846 base pair gene was spliced into the PET28c bacterial expression vector containing an inducible isopropyl-β-D-thiogalactopyranoside T7 promoter and a kanamycin selection gene (Fig. 1A). To verify that the dCD133KDEL gene had been cloned correctly and in frame, DNA sequence analysis was done at the University of Minnesota BioMedical Genomics Center. The CD133scFv was separately cloned into the PET28c bacterial expression vector and produced to determine CD133 expression of various cell lines in flow cytometry studies.

Purification of CD133scFv and dCD133KDEL
Purification of CD133 scFv and dCD133KDEL was done as described previously (26). Briefly, each protein was expressed and purified from inclusion bodies using a Novagen pET expression system (Novagen) followed by a 2-step purification consisting of ion exchange fast protein liquid chromatography (Q sepharose Fast Flow, Sigma) and size exclusion chromatography (Hiload Superdex 200, Pharmacia). The purified protein was then analyzed by SDS-PAGE and stained with Commasie Brilliant Blue to determine purity.

Cell lines and culturing technique
UMSCC-11B is a squamous cell carcinoma cell line that was derived from larynx tumor following chemotherapy (27). UMSCC-11B-luc was transfected using a luciferase reporter construct and was maintained under 10 μg/mL of blastocidin. Cells were transfected using Invitrogen’s Lipofectamine Reagent. NA-SCC is another squamous cell carcinoma line isolated from a tongue tumor (28). Both lines were obtained from Dr. Frank Ondrey (University of Minnesota) who originally obtained them from their originator, Dr. Thomas E. Carey, Department of Otalaryngology-Head and Neck Surgery, University of Michigan in 2009. NA and UMSCC cell lines were authenticated this year by STR testing done by the Fragment Analysis Facility, John Hopkins University. Caco-2 (a colorectal carcinoma) and HEK293 (a human embryonic kidney cell line) were obtained from the American Type Culture Collection and have not been authenticated, but were positive for the appropriate markers. Only cells that were greater than 90% viable were used for experimentation.

Flow cytometry and CD133+ cell enrichment
Flow cytometry was done using a fluorescence-activated cell sorting (FACS) caliber at the University of Minnesota’s Flow Cytometry Core Facility. Fluorescein isothiocyanate (FITC)-labeled antibodies were used and results were analyzed using FLOWJO. Sorting was done using a magnetic bead selection kit following manufacturer’s instructions (Stem Cell Technologies). Briefly, cells were concentrated to 2 × 10^6 cells/mL. FITC-labeled CD133scFv was added to cells at a concentration of 1.0 μg/mL and incubated for 15 minutes. The EasySep FITC Selection Cocktail was then added followed by the EasySep magnetic nanoparticles after a 15-minute incubation. The cells were mixed and then placed within the magnet. Unbound cells were eluted and bound cells collected.

Time course viability assays
Trypan blue viability assays were done by plating 10,000 cells/well into 24-well plates. Toxin and media were replaced daily at 0.01 nmol/L for NA-5CC cells or 0.03 nmol/L for UMSCC-11B cells. Wells were harvested every other day using trypsin digestion and counted on a hemocytometer via trypan blue staining. Untreated wells typically became confluent around day 8. For the time course viability assays on CD133-negative sorted cells, UMSCC-11B cells were incubated with CD133 scFv-FITC and sorted using a FACSARIA at the University of Minnesota’s Flow Cytometry Core Facility. The 20,000
cells/well were sorted into 24-well plates. The rest of the time course viability assay was done as described above.

**Hematopoietic colony-forming unit assays**

Two types of assays were done: short-term (2 weeks) hematopoietic colony-forming assays (CFC) and long-term (5 weeks) culture assays (LT-CIC; ref. 29). For CFC assays, progenitor cells were sorted from human umbilical cord blood (UCB). CD34+ cells were sorted using magnetic selection and collected in MethoCult GF+H4435 (StemCell Technologies; catalogue no. 04435) consisting of 1% methylcellulose, 30% FBS, 1% bovine serum albumin, 50 ng/mL stem cell factor, 20 ng/mL granulocyte–macrophage colony-stimulating factor, 20 ng/mL interleukin (IL)-3, 20 ng/mL, IL-6, 20 ng/mL granulocyte colony stimulating factor, and 3 U/mL erythropoietin. Targeted toxin was added at the desired dosages and cultures were incubated at 37°C, 5% CO2 for 2 weeks and then scored for colony-forming units according to standard criteria after 2 weeks. CFU-GEMM, CFU-GM, and BFU-E were counted individually. For LT-CIC assays, cells were cultured in commercial Myelocult media from StemCell Technologies with M2-10B4 stromal cells. Colonies were quantitated after 5 weeks.

**Tumor initiation experiment**

For the tumor initiation study, UMSCC-11B squamous carcinoma cells were injected into the right flank of nude mice at a concentration of 300,000 cells/mouse. Tumor size was measured using digital calipers and tumor volume (width × length × height) was calculated. Before injection, cells were treated with 0.03 nmol/L of either dCD133KDEL or the nonspecific toxin, CD22-KDEL. A third group of cells was left untreated and unsorted. For the sorted group, cells were sorted for CD133 expression with a magnetic bead kit as previously described, plated overnight, harvested, and injected the following day.

**Tumor treatment study and imaging**

For experiment 1, nude mice were injected with 3 million UMSCC11B-luc cells into the right flank of tumor. Tumors were treated with dCD133KDEL, CD3CD3KDEL, or PBS starting on day 8 when they had become palpable. A single course of treatment consisted of an injection of 20 μg of drug given every other day (Monday, Wednesday, Friday) and mice were given a total of 8 injections. Controls were treated with PBS. Tumor volume was measured with calipers over time. For experiment 2, nude
mice were injected with 6 million UMSCC-11B-luc cells in the right flank. Tumors were treated starting on day 10 postinoculation. Treatment was done as in experiment 1, except mice received 16 injections. Also, an anti-B cell control, CD19KDEL, was used. Tumor volume was calculated before treatment. Because tumors were marked with a luciferase reporter gene, mice were imaged to determine their bioluminescent activity as described previously (23). Briefly, mice were injected with 100 μL of 30 mg/mL luciferin substrate 10 minutes before imaging and then anesthetized via inhalation of isoflurane gas. The mice were then imaged using the Xenogen Ivis 100 imaging system and analyzed with Living Image 2.5 software (Xenogen Corporation). Five-minute exposures were made and units for the regions of interest were expressed as photons/sec/cm²/sr. For experiment 1, tumor treatment was stopped on day 24. For experiment 2, treatment was stopped on day 45.

**Histology**

Suspected tumor-free survivors were sacrificed and histology evaluation was done at the termination of the experiment. Liver, kidney, and skin (from the tumor site) samples were taken. These samples were embedded in OCT compound (Miles, Elkhart), snap frozen in liquid nitrogen, and stored at −80°C until sectioning. Sections of the tissues were cut, thawed, and mounted on glass slides. Slices were fixed for 5 minutes in acetone and then stained with hematoxylin and eosin.

**Determining immunogenicity of deimmunized CD133KDEL**

To determine whether mutated CD133KDEL elicited less of an antitoxin response than nonmutated parental toxin, female Balb/c (n = 5/group) were injected intraperitoneally once weekly with 0.25 μg of either deimmunized CD133KDEL or nonmutated parental PE38KDEL toxin. Each week, 5 days after injection, the mice were bled via face vein. Serum was isolated using centrifugation, frozen, and the level of anti-PE38KDEL IgG in each sample was measured by ELISA. Briefly, PE38KDEL was bound 96-well microtiter plates at a concentration of 5 μg/mL overnight at 4°C. Unbound protein was washed away with PBS-T and blocking was done for 1 hour with 5% milk/PBS-T. Serum samples were diluted appropriately and 100 μL of each was added to wells in triplicate. After 3 hours, each well was washed with PBS-T. Peroxidase-conjugated rabbit antimouse IgG (Sigma) was added to each well. After 2 hours, samples were washed and o-phenylendiamine dihydrochloride substrate was added. After 30 minutes, the absorbance at 490 nm was measured using a microplate reader. Quantification of actual anti-PE38KDEL IgG present in each sample was determined by comparing the absorbance values in each well to a standard curve prepared using M40-1 monoclonal anti-PE38KDEL antibody from Dr. Robert Kreitman (NIH, Bethesda, MD).

**Results**

**Anti-CD133 scFv construct binds selectively**

SDS-PAGE analysis showed that following ion exchange and size exclusion chromatography, dCD133KDEL was greater than 95% pure (Fig. 1B).

To establish that the anti-CD133 scFv construct recognized the CD133 receptor, HEK293 cells were transfected with a gene encoding the CD133 receptor. Figure 1C is a histogram plot showing a high degree of reactivity of the anti–CD133-FITC with the transfected cells. Anti–CD22-FITC, however, did not bind the transfected cells. Interestingly, it seems that there is a high intensity and a low intensity peak indicating populations with high and low antigen densities. Table 1 shows the expression of CD133 on various cell populations measured by flow cytometry. UMSCC-11B and NA-SCC cell lines were 4.0% and 5.9% CD133+, respectively. These cells were tested with anti-CD19-FITC as a negative control and were less than 1.2% CD19+. Caco-2, a human colorectal cell line, was tested as positive control and was 79% positive for CD133 expression. These numbers are from representative experiments that have been duplicated at least 3 times each. Together, these findings showed that the CD133 scFv is specific to the CD133 receptor that is selectively expressed on a small subpopulation of cancer cells.

**Time course viability assays show proliferation inhibition**

If CD133 is a marker for CSC, then selective killing of this subpopulation of cells should be sufficient to inhibit proliferation (3). To test this, dCD133KDEL was added to cells in a trypan blue viability time course assay to determine the number of viable cells. As seen in Fig. 2A, the proliferation of UMSCC-11B cells was almost

<table>
<thead>
<tr>
<th>Table 1. CD133 expression on cancer cell lines</th>
<th>% Positive cells</th>
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<tr>
<td>Marker</td>
<td>UMSCC-11B</td>
</tr>
<tr>
<td>CD133+</td>
<td>4.0</td>
</tr>
<tr>
<td>EpCAM+</td>
<td>98.0</td>
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<tr>
<td>CD45+</td>
<td>0.5</td>
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<td>CD19+</td>
<td>0.7</td>
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NOTE: CD133 expression was measured on various human carcinoma lines. The anti-CD133scFv was tagged with FITC and then reacted with the head and neck cancer cell lines NA-SCC and UMSCC-11B. The Caco-2 colorectal carcinoma was included as a positive control cell line because it is known to overexpress CD133. Controls included cells reacted with anti-EpCAM-FITC, anti-CD45-FITC, and anti-CD19-FITC. Expression of CD45 and CD19 is mostly restricted to normal malignant hematopoietic cells and thus served as negative controls.
Effect on human progenitor colony formation

CD34+ UCB cells are considered a rich source of normal hematopoietic progenitor cells. To determine the effect of dCD133KDEL on the development of hematopoietic cells derived from CD34+ progenitors, sorted CD34+ cells were treated with drug and evaluated in 2 standardized assays of hematopoietic stem/progenitor cell survival and function. Notably, the UCB CD34+ cells used for this study were first studied using 2-color flow cytometry and 67% coexpressed CD34 and CD133, whereas 24% were CD34+CD133− and none of the cells were CD34−CD133+. dCD133KDEL drug concentrations were selected based on the results in Fig. 2. Initially, studies of hematopoietic colony formation as a measure of hematopoietic progenitor cell survival and function. Notably, the UCB CD34+ cells used for this study were first studied using 2-color flow cytometry and 67% coexpressed CD34 and CD133, whereas 24% were CD34+CD133− and none of the cells were CD34−CD133+. CD133+ cells efficiently initiate tumors

CD133+ cells efficiently initiate tumors (16, 17). Thus, a tumor initiation study was done to determine whether cells pretreated with dCD133KDEL would form tumors in vivo (Fig. 4). UMSCC-11B cells were pretreated with 0.03 nmol/L of dCD133KDEL for 6 days before inoculation in the flanks of nude mice. Control mice were injected with either untreated cells or cells treated with a nonspecific toxin targeting CD3. UMSCC-11B cells were also enriched for CD133+ cells using magnetic bead separation before inoculation. When cells were sorted using the magnetic bead separation and run using flow cytometry, the expression of CD133+ cells increased 4-fold (data not shown). As seen in Fig. 4, cells treated with dCD133KDEL before injection had the lowest incidence of tumor formation (1 of 4). The single tumor that formed was markedly smaller than tumors formed by the control or CD133-enriched cells. CD133+ enriched cells formed tumors at the highest incidence, formed earlier, and grew much larger than control tumors. These results further indicate that we are killing CSC and support the fact that CD133 is a CSC marker in head and neck carcinomas.

Tumor treatment studies show impressive tumor regression

In experiment 1, animals with palpable tumors were given intratumoral injections of 20 μg/injection of dCD133KDEL. Visual measurement of tumor progression with calipers showed that tumors regressed and were gone by day 24 when treatment was stopped and control tumors progressed (Fig. 5A). The same animals
were imaged in real time and the images showed that control tumors given either PBS (M1-M3) or treated in an identical fashion with anti-T cell CD3CD3KDEL (M4, M5) did not regress (Fig. 5B). No tumor was detected by day 24 in treated mice indicating that real-time imaging correlated with caliper data. In experiment 2 (Fig. 5C), animals were given twice the number of tumor cells, so tumors grew faster, and treatment was delayed until day 10. Although tumors grew more aggressively, all mice treated with dCD133KDEL responded to treatment. Mice treated with control CD19KDEL did not. Together, the combined experiments showed that by killing the CD133+ subpopulation of cells, the tumors were unable to generate more cells and eventually regressed.

Histologic analysis was done on tumors from experiment 1. Figure 5D shows a skin section from a mouse, taken on day 59, in which an epithelial-like tumor is prominent. The tumor is robust and vascularized. Other sections revealed necrosis. Also shown is healthy skin from a mouse that was treated intratumorally with dCD133KDEL, which prevented tumor elimination. The skin pathology looks mostly normal with intact hair follicles, although some evidence of inflammation is still present. The section is part of the histologic analysis that confirms the animal was tumor free. The livers and kidneys of both treated and nontreated animals showed no evidence of tumor indicating that it did not metastasize to these organs.

Antitoxin levels greatly reduced in dCD133KDEL immunized mice

To ensure that we had effectively deimmunized dCD133KDEL, BALB/c mice were immunized with either mutated dCD133KDEL or parental nonmutated PE38KDEL. Mice (n = 5/group) were immunized intraperitoneally once a week and were bled on day 63. Despite 9 weekly immunizations with drug (0.25 μg/injection), the dCD133KDEL group had significantly lower serum antitoxin levels than the animals immunized with parental toxin (Fig. 6). Generally, deimmunization resulted in an average 90% reduction in antitoxin levels.

Discussion

The major contributions of this manuscript are the development of a novel-targeted toxin, dCD133KDEL, which targets CD133 tumor-initiating cells and its efficacy against 2 head and neck carcinoma lines. dCD133KDEL is a new and powerful reagent for 3 main reasons. The drug (i) targets only a small subpopulation of the total tumor, (ii) has been successfully deimmunized bypassing a major clinical problem with targeted toxins, and (iii) has a mechanism of action unlike typical chemotherapy agents. Targeted toxins function by binding to cell surface receptors, internalizing, and enzymatically inhibiting protein synthesis (30). CD133 is readily internalized rendering it an excellent marker for a targeted
Toxin (14). The fact that we observed heterogenous peaks in CD133 expression in transfected HEK cells in Fig. 1C supports the argument that CD133 cycles and internalizes.

Bonnet and Dick reported the first CSC subpopulation in leukemia in 1997 (31). Since then investigators have validated the presence of CSC subpopulations not only in leukemia, but in many carcinomas as well. CD133 in particular has been identified as a CSC marker in these carcinomas (6, 10–13). Wei and colleagues showed in their work that CD133 is a marker for the head and neck laryngeal cancer line HEP-2. In their study, they showed that CD133+ sorted cells initiated tumors and uniquely possessed clonogenic capacity when compared with CD133− cells (16). We were able to confirm CD133 as a marker for tumor initiation cells in the current study with a different head and neck cell line UMSCC-11B.

Also unique to our study is the use of a new monoclonal antibody (clone 7) that binds all forms of the CD133 receptor (18). This is important because commercial antibodies currently available recognize an epitope that can be masked upon differentiation (32). In contrast, the scFV from clone 7 was used in our construction of dCD133KDEL recognizes only the CD133 peptide backbone, avoiding the issue of epitope masking or differential glycosylation. Specificity was determined by showing that our anti-CD133 scFV recognized cells transfected with DNA encoding the CD133 receptor. Furthermore, head and neck cell lines contained subpopulations of CD133 expressing cells at similar levels to other head and neck cell lines described in the literature (16, 17, 33). When dCD133KDEL was tested in vitro, we discovered that it selectively inhibited cancer cell expansion in both cell lines. We found that about 5% of the UMSCC-11B head and neck cancer cells used in the study were Annexin V positive following CD133KDEL treatment indicating apoptotic death. Since flow studies also showed that about 5% of the UMSCC-11B population was CD133+, this suggests an apoptotic death for CD133 cells. We also attempted dual staining to confirm that the same population that was Annexin positive was CD133 positive. However, these mechanistic studies proved complicated since earlier treatment with dCD133KDEL interfered with our ability to later recognize and quantitate CD133+ cells. We attributed this difficulty to either blocking interference or the high modulation rate of CD133.

Because tumor initiation is a hallmark of CSC, UMSCC-11B tumor cells were pretreated with dCD133KDEL and transplanted into nude mice. These cells formed the lowest incidence of tumors compared with controls, while cells enriched for CD133+ expression formed the largest tumors at the fastest rate. Furthermore, in 2 separate studies tumor cells were injected into the flanks of nude mice and when palpable were directly treated with dCD133KDEL. All treated tumors regressed over time, unlike control tumors and all treated animals were impressively tumor free after 79 days. We favor the explanation that dCD133KDEL acts to inhibit the self-renewing ability of CD133−/CSC. However, it is possible that dCD133KDEL has a bystander killing effect but this was not supported by our in vitro studies showing that dCD133KDEL did not kill CD133−/CSC.

Several studies suggest the existence of another CSC population in some tumors that is CD133−/CSC− (34, 35). For example, Chen and colleagues discovered CD133−/CSC− cells within glioblastoma primary tumors that had the capability to self-renew and support long-term growth in vitro and initiate tumors in vivo (35). dCD133KDEL may be a helpful biological tool in validating the existence of these cells and in helping to develop more inclusive therapies that may target all CSC and progenitors simultaneously.

The development of a novel targeted toxin that selectively targets CSC may have unique implications for the ubiquitous problem of carcinoma drug resistance and subsequent relapse. Several studies have shown that CSC are resistant to current chemotherapeutic agents (5, 6, 10, 36, 37). Because targeted toxins work by a different mechanism and because they selectively kill CSC, dCD133KDEL may possess the unique ability to target the very cells responsible for drug resistance. Adding a targeted toxin as an adjunct to chemotherapy has been shown to be more effective than using either therapy.
alone (38, 39). Studies are underway to determine whether dCD133KDEL may be a useful adjunct to chemotherapy. One of the major limitations of targeted toxins in clinical therapy is immunogenicity. Patients will start developing antibodies to the toxin and thus the number of treatments is limited. To address this problem, we mutated 7 immunogenic epitopes that account for the majority of antibodies produced against this form of PE toxin. We then used this mutated construct to create our fusion protein. As a result, we showed that even after 9 immunizations there is very little antibody produced against the toxin moiety of dCD133KDEL and 40% of the animals had no antibody response at all.

Drug safety issues regarding the affects of dCD133KDEL on normal human hematopoietic progenitor cells are important and need to be addressed before any therapeutic consideration. Therefore, we isolated CD34+ cells from UCB an established source of normal human hematopoietic stem cells, cultured them with dCD133KDEL and then determined their ability to form various hematopoietic colonies in established CFU (colony-forming unit) assays. To be sure, we changed media containing drug weekly and cultured the cells in long-term colony initiation assays for 5 weeks. Only toxin alone inhibited survival of hematopoietic progenitor cells. These results indicate that although the UCB

Figure 5. Tumor treatment studies show tumor regression. In A, tumor size was measured for experiment 1 using digital calipers. A student’s paired t test was done on the average tumor size over the course of the experiment between the control and treated groups. The 2 curves are significantly different with a P value of 0.0001. Experiment 1 (B) and experiment 2 (C) are shown with tumor bioluminescent and images showing that dCD133KDEL inhibits the progression of UMSCC-11B-luc nude mice flank tumors. For experiment 1 (B), 3 million cells were injected and tumors were treated with dCD133KDEL (M6–M10) while controls were treated intratumorally with either PBS (M1–M3) or the anti-T CD3CD3KDEL (M4–M5) starting on day 8. This also shows that dCD133KDEL inhibits the progression of flank tumors and correlates with the caliper data. The arrows indicate where treatment (Tx) began and ended. For experiment 2 (C), tumors were induced by injection of 8 million cells. Intratumoral treatment was begun on day 10. Tumors were treated exactly as they were in experiment 1 except mice were given 16 injections instead of 8. Controls were untreated or treated with anti-B cell CD19KDEL. Animals were imaged weekly for both experiments. Bioluminescence intensity was measured. D, histology photomicrograph shows skin tissue taken from the tumor injection site of a representative tumor-free mouse from experiment 1. As a control, a photo is shown of skin tissue taken from a PBS-treated control mouse. Tumor is clearly visualized in controls, but absent in the drug-treated mouse.
CD34+ cells coexpress CD133, these cells are resistant to treatment with dCD133KDEL. These findings could be explained by multiple normal stem cell populations that are CD133− since 24% of the starting stem cells were CD34+CD133−. Rutella and colleagues discovered a stem cell population in human cord blood that was CD133− and still capable of differentiating into multiple cell types (40). Also, Surronen and colleagues showed that CD133+ cells can be generated from normal CD133− cells (41). Perhaps dCD133KDEL destroyed normal CD133+ cells and replacements emerged from the CD133− fraction. Alternatively, CD34+CD133+ cells may be metabolically quiescent enough or have other drug resistance mechanism to prevent significant killing by dCD133KDEL. Finally, the expression level of CD133 on tumor cells may be greater than the expression level on normal progenitors (42). This could allow for the observed selective killing of CSC over normal stem cells. Sehl and colleagues used mathematical modeling to conclude that in order for a treatment to be safe it must be highly selective and be able to target quiescent CSCs (43) and because dCD133KDEL is selective and because cells do not have to be actively cycling for targeted toxins to be taken up and effectively cause apoptosis, dCD133KDEL warrants further investigation.

Several cell populations in the body express CD133 and therefore may be potential targets for the toxin, causing either short-term or long-term health effects. Thus, extensive toxicity studies will be necessary to validate a CD133 targeted drug for clinical use. These studies will not be trivial. CD133 expression on normal cells has proven somewhat complicated since CD133 has been shown to undergo post translational modulation such that mRNA and internal protein expression does not correlate with the surface expression (44). Also, the commercial antibodies such as AC133 only bind certain forms of the receptor (18, 32). Furthermore, studies in an ontarget model closer to humans than rodents may be necessary. In summary, we developed a novel deimmunized targeted toxin that selectively binds the CSC marker CD133. dCD133KDEL impressively inhibited cell proliferation in vitro, decreased tumor initiation, does not kill normal human hematopoietic progenitor cells, and caused complete tumor regression in an accepted model of human head and neck cancer which is a xenograft model.

This work represents the development of a new cancer therapeutic that functions by selectively targeting the minority CSC subpopulation within the tumor. We believe dCD133KDEL warrants further study as a possible solution for drug-resistant relapse in human carcinoma.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

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