Therapeutic Discovery

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). 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 toxins 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 A (PE38) (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 NotI 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-b-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 Commassie 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 blasticidin. 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 Otolaryngology-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) caliper 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 × 10⁶ 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 Easy-Sep 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-SCC cells or 0.03 nmol/L for UMSCC-11B cells. Wells were harvested every other day using tyospin 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 FACS ARIA 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 x length x 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. 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, Elkhark), 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-phenylenediamine 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

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<th>Marker</th>
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<th>Caco-2</th>
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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.
completely ablated, while a nonspecific control toxin targeting CD22 did not inhibit cell proliferation. dCD133KDEL had the same effect on NA-SCC cells, another head and neck carcinoma line (Fig. 2B). To determine whether dCD133KDEL was having off-target effects, we sorted UMSCC-11B cells for CD133− cells and conducted a time course viability assay. As seen in Fig. 2C, the targeted toxin showed no effect on the CD133−-sorted cells. These plots show representative experiments that have each been reproduced.

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, another CSC marker in head and neck carcinomas. We conducted a time course viability assay. Media and drug were renewed weekly.

Figure 2. Trypan blue viability studies show that dCD133KDEL selectively inhibits head and neck cancer cells over time. UMSCC-11B (A) or NA-SCC (B) cells were incubated with dCD133KDEL and viability was determined over time by directly counting cells growing in tissue culture wells using a trypan blue vital stain. Live cells exclude the dye and dead cells incorporate it. CD22KDEL, CD19KDEL (anti-B cell), and CD3CD3KDEL (anti-T cell) targeted toxins were included as negative controls. Media and toxin were replenished daily. In C, UMSCC-11B cells were sorted for CD133− cells and immediately incubated with dCD133KDEL to determine nonspecific killing. dCD133KDEL did not inhibit CD133+. UMSCC-11B cells treated immediately following sorting. Error bars were calculated for all data points, but are not visible in some instances because error margins were very slight.

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 injected with untreated cells or control toxin (dCD133KDEL pretreatment) and tumors were measured daily. The targeted toxin showed no effect on the CD133−-enriched cells. CD133− cells efficiently initiate tumors
CD133+ CSC possess the unique ability to 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.

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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 growth and eventually led to complete 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
Fig. 1C supports the argument that CD133 cycles and internalizes. dCD133KDEL recognizes only the CD133 peptide back-end of the receptor (14). The fact that we observed heterogeneous peaks in CD133 expression in transplanted 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 CSC marker for the head and neck laryngeal cancer line HEP-2. In their study, they showed that about 5% of the head and neck cancer cells used in the study were Annexin V positive following CD133 treatment indicating apoptotic death. Since flow studies also showed that about 5% of the UMSCC-11B head and neck cancer cells used in the study were CD133+, this suggests an apoptotic death for CD133 cells.

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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
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− (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.

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 an xenotransplant flank 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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No potential conflicts of interest were disclosed.

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