Construction of an Immunotoxin, D2C7-(scdsFv)-PE38KDEL, Targeting EGFRwt and EGFRvIII for Brain Tumor Therapy

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Abstract

Purpose: The EGF receptor gene (*EGFR*) is most frequently amplified and overexpressed, along with its deletion mutant, *EGFRvIII*, in glioblastoma. We tested the preclinical efficacy of the recombinant immunotoxin, D2C7-(scdsFv)-PE38KDEL, which is reactive with a 55-amino acid (AA) region present in the extracellular domain of both EGFRwt (583-637 AAs) and EGFRvIII (292-346 AAs) proteins.

Experimental Design: The binding affinity and specificity of D2C7-(scdsFv)-PE38KDEL for EGFRwt and EGFRvIII were measured by surface-plasmon resonance and flow cytometry. *In vitro* cytotoxicity of D2C7-(scdsFv)-PE38KDEL was measured by inhibition of protein synthesis in human EGFRwt-transfected NR6 (NR6W), human EGFRvIII-transfected NR6 (NR6M), EGFRwt-overexpressing A431-epidermoid-carcinoma, and glioblastoma xenograft cells (43, D08-0493MG, D2159MG, and D270MG). *In vivo* antitumor efficacy of D2C7-(scdsFv)-PE38KDEL was evaluated using 43, NR6M, and D270MG orthotopic tumor models.

Results: The K_D of D2C7-(scdsFv)-PE38KDEL for EGFRwt and EGFRvIII was 1.6×10^{-9} mol/L and 1.3×10^{-9} mol/L, respectively. Flow cytometry with NR6W and NR6M cells confirmed the specificity of D2C7-(scdsFv)-PE38KDEL for EGFRwt and EGFRvIII. The D2C7-(scdsFv)-PE38KDEL IC$_{50}$ was 0.18 to 2.5 ng/mL on cells expressing EGFRwt (NR6W, A431, 43, and D08-0493MG). The D2C7-(scdsFv)-PE38KDEL IC$_{50}$ was approximately 0.25 ng/mL on EGFRvIII-expressing cells (NR6M) and on EGFRwt- and EGFRvIII-expressing glioblastoma xenograft cells (D2159MG and D270MG). Significantly, in intracranial tumor models of 43, NR6M, and D270MG, treatment with D2C7-(scdsFv)-PE38KDEL by convection-enhanced delivery prolonged survival by 310% ($P = 0.006$), 28% ($P = 0.002$), and 166% ($P = 0.001$), respectively.

Conclusions: In preclinical studies, the D2C7-(scdsFv)-PE38KDEL immunotoxin exhibited significant potential for treating brain tumors expressing EGFRwt, EGFRvIII, or both. *Clin Cancer Res; 19(17); 4717–27.* ©2013 AACR.

Introduction

Gliomas are the most common primary tumors of the central nervous system (1). The most frequent and most malignant type of glioma, glioblastoma multiforme, accounts for approximately 16% of all brain tumors in the United States (2). Current treatment for patients with glioblastoma includes surgery followed by radiation and che-

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motherapy; however, the median survival for patients with glioblastoma until the early 1990s was less than a year (3, 4). Recent trials incorporating temozolomide or the monoclonal antibody (mAb) bevacizumab have resulted in only modest extensions in survival (5, 6). Clearly, new and more efficient therapeutic approaches are needed to improve survival of patients with glioblastoma.

In the past decade, mAb-based clinical trials have increasingly focused on genetically engineered single-chain variable-region antibody fragments (scFv) consisting of the heavy- and light-chain variable regions (V_H and V_L) fused to immunotoxins; these scFv-immunotoxins target the antigens expressed specifically by brain tumor cells. Because an scFv-immunotoxin fusion protein is smaller than an intact immunoglobulin (IgG), it should have greater tumor penetration and, therefore, lead to enhanced therapeutic efficacy when delivered intrathecally, intratumorally, or intracerebrally (7–11).

The EGF receptor (EGFR), a 170 kDa, transmembrane receptor tyrosine kinase (RTK), is frequently overexpressed in a wide variety of human cancers (12), including brain tumors (13, 14). *EGFR* is the most frequently amplified gene

Translational Relevance

Glioblastoma multiforme is the most malignant and most frequently occurring brain tumor. The epidermal growth factor receptor (EGFRwt) and its mutant, EGFRvIII, which is not found on normal tissues, are overexpressed in 60% to 90% and 58% to 61% of all glioblastomas, respectively. D2C7-(scdsFv)-PE38KDEL is a recombinant immunotoxin, targeting both EGFRwt and the tumor-specific EGFRvIII.

We show that D2C7-(scdsFv)-PE38KDEL has significant antitumor activity due to its affinity for EGFRwt and EGFRvIII. This immunotoxin is efficacious in *in vitro* cytotoxicity assays and in *in vivo* orthotopic models of glioblastoma xenograft cells that express EGFRwt, EGFRvIII, or both.

The dual-specific activity of this new toxin should translate well into the clinical setting. The specificity and high binding affinity toward both targets indicate that D2C7-(scdsFv)-PE38KDEL is likely to show greater efficiency in treating brain tumors than monospecific therapeutics. Funding has been awarded for phase I and II clinical trials of D2C7-(scdsFv)-PE38KDEL in patients with glioblastoma.

in glioblastoma (15) and *EGFR* amplification defines the classical glioblastoma subtype (16); in contrast, the level of EGFR in normal brain is undetectable or extremely low (12). Correlating with the gene amplification, the protein is overexpressed in approximately 60% to 90% of glioblastoma cases. Even in the absence of gene amplification, protein overexpression has been observed in 12% to 38% of patients having glioblastoma (17), which may be due to aberrant translational and posttranslational mechanisms. Preclinical studies have shown that EGFR activation, in addition to protecting tumor cells from apoptosis, also induces several tumorigenic processes, including proliferation, angiogenesis, and invasiveness (18).

EGFR amplification is often associated with gene rearrangements. Several *EGFR* deletion mutants have been identified, the most common being *EGFRvIII*, which is present in 67% of glioblastomas with *EGFRwt* amplification (19). *EGFRvIII* contains a deletion of exons 2–7 of the *EGFR* gene, (20) and this in-frame deletion creates a novel glycine residue at the fusion junction at position 6, between amino acid residues 5 and 274, thus generating a tumor-specific epitope that is expressed specifically on tumor cells, but not on normal tissues. EGFRvIII is a constitutively active RTK that is not further activated by EGFR ligands (21). Like its wild-type counterpart, EGFRvIII is widely expressed in malignant gliomas (22) and carcinomas, including that of the head and neck (23) and breast (24). Overexpression of EGFRvIII induces resistance in glioma cells to radiation and chemotherapy (25).

Several anti-EGFR mAbs are in clinical trials for various human cancers, including head and neck, colorectal, pan-

creatic, lung, renal cell, and prostate carcinoma or high-grade glioma (7, 26). The anti-EGFRwt mAbs EGFR1, H17E2, and 425 were the first to be introduced in targeted radiotherapy trials that involved systemic injection of radiolabeled mAbs in patients with malignant gliomas (27–29). Furthermore, in a phase I clinical trial with TP-38, a recombinant EGFR ligand (TGF-α) *Pseudomonas* exotoxin fusion protein, an overall median survival of 23 weeks for all 20 patients with glioblastoma enrolled was observed (30). In addition, a recombinant human EGF diphtheria toxin fusion protein (DT-EGF) inhibited tumor growth in an *in vivo* xenograft model with EGFR-expressing U87MG glioma cells, and 75% of the treated animals remained tumor-free 60 days posttreatment (31).

Several mAbs and scFv constructs specific for EGFRvIII, including L8A4, Y10, MR1, MR1-1, and 14E1, are well described in previous studies (24, 32–34). Among the various antibody constructs, MR1-1 scFv, derived from a mouse scFv library, has significant potential (32, 33). A phase I clinical study with the MR1-1 *Pseudomonas* immunotoxin delivered by convection-enhanced delivery (CED), is currently underway at Duke University (Durham, NC) for treating patients with EGFRvIII-expressing glioblastoma tumors (http://clinicaltrials.gov/ct2/show/NCT01009866).

Because of the high prevalence of EGFRvIII mutation in tumors that have *EGFRwt* amplification (19), it would be advantageous to have antibodies that could target both antigens for glioblastoma therapy. Co-targeting these two antigens could promote greater killing of tumor cells than that which is achieved by antibodies specific for a single antigen. Cetuximab, an unarmed EGFRwt- and EGFRvIII-reactive antibody, has shown limited activity (progression-free survival of <6 months) in a phase II trial in recurrent, high-grade glioma in patients with *EGFRwt* amplification (35). Our study focuses on D2C7, a novel mAb that reacts with both EGFRwt and EGFRvIII proteins (36). In comparison with the established specific mAbs (anti-EGFRwt mAb, EGFR1, or anti-EGFRvIII mAb, L8A4), D2C7 showed a significantly higher tumor localization in tumors expressing EGFRwt or EGFRvIII proteins (36). Significantly, in immunohistochemical analysis of 101 adult glioblastoma samples, the D2C7 mAb positively stained virtually all cells in 100% (50/50) of the samples that had amplification of the *EGFRwt* gene and in 76% (39/51) of the cases without this amplification (36).

Here, we summarize the *in vitro* and *in vivo* results of our investigation of D2C7-(scdsFv)-PE38KDEL, a recombinant scFv immunotoxin that binds to both EGFRwt and EGFRvIII and has potential clinical application for the therapy of brain tumors expressing these proteins.

Materials and Methods

Cell lines

The cell lines used were, the human epidermoid-carcinoma cell line, A43l (expressing EGFRwt), the murine Swiss 3T3 fibroblast cell line NR6 transfected with human EGFRwt (NR6W; ref. 21), and human EGFRvIII (NR6M;

ref. 21). The parental murine Swiss 3T3 fibroblast cell line, NR6 (kindly provided by Dr. Harvey Herschman, University of California, Los Angeles, CA), which lacks expression of murine or human EGFRwt and EGFRvIII, was used as control. All cell lines were cultured in complete zinc option (ZO)-10% FBS [Improved Modified Eagle Medium ZO (, Richter's Modification, Cat. No. 10373-017) liquid; Invitrogen] and passed at confluence with 0.05% trypsin–EDTA (Invitrogen). All cell lines were authenticated by periodic morphologic assessment and by testing for mycoplasma infection.

D2C7 epitope mapping by ELISA

Different concentrations (0.5, 0.25, 0.125, and 0.0625 µg/mL in 0.1 mol/L carbonate buffer pH 10) of Nus-Tag EGFRvIII ECD deletion constructs together with the Nus-Tag protein alone were added to 96-well plates and incubated for 1 hour at 37°C. The plates were then blocked with SuperBlock buffer (Pierce) for 30 minutes at 37°C and incubated with 1.0 µg/mL of D2C7 mAb for 1 hour at 37°C. The plates were then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody for 1 hour at 37°C, followed by the addition of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (Pierce). The reaction was stopped by the addition of stop solution (Pierce), and the optical density was measured at 450 nm.

Disaggregation of xenograft tumor samples

Xenograft tissue derived from glioblastoma samples D270MG (expressing EGFRwt and EGFRvIII), D2159MG (expressing EGFRwt and EGFRvIII), 43 (expressing EGFRwt; kindly provided by Dr. C. David James, University of California, San Francisco, CA), and D08-0493MG (expressing EGFRwt), was finely minced and digested with 100 µg Liberase (Roche) at 37°C for 10 minutes. The dissociated cells were filtered and the reaction was inhibited with the addition of 5% human serum albumin (HSA) solution. The cells were washed with ZO medium, further treated with Ficoll–Hypaque to remove any red blood cells, and then washed again in ZO medium.

Preparation of recombinant immunotoxins

D2C7 and P588 immunotoxins were generated by fusing the specific scFv with the sequences for domains II and III of *Pseudomonas* exotoxin A (PE38) according to the protocol described in a previous publication (37). The specific scFv immunotoxin was expressed under control of the T7 promoter in *Escherichia coli* (*E. coli* BL21 λ DE3; Stratagene). All recombinant proteins accumulated in the inclusion bodies. The immunotoxins were then reduced, refolded, and further purified as monomers (64 kDa) by ion-exchange and size-exclusion chromatography to more than 95% purity, as described in another study (37).

Flow cytometry

Indirect fluorescence-activated cell sorting (FACS) analysis was carried out with the D2C7-(scdsFv)-PE38KDEL immunotoxin. Briefly, 1×10^6 cells (NR6, NR6W, or NR6M) were suspended in 500 µL of PBS pH 7.4 (Invitrogen) containing 5% FBS (5% FBS/PBS; Invitrogen). The D2C7-(scdsFv)-PE38KDEL immunotoxin or negative control, P588-(scdsFv)-PE38KDEL, was added to the cells at a concentration of 10 µg/mL, and the samples were incubated for 40 minutes at 4°C. After washing, cells were incubated with rabbit anti-*Pseudomonas* exotoxin A antibody (Sigma) followed by goat anti-rabbit-IgG-FITC antibody (Zymed). Stained cells were washed with 1× PBS and analyzed on a Becton Dickinson FACSort instrument equipped with Cell-Quest software (BD Biosciences). Glioblastoma (43 and D2159MG) xenograft cells (5×10^5) were suspended in 500 µL of 5% FBS/PBS. Human CD133-APC (AC133 and 293C3; Miltenyi Biotec) and D2C7-AF488 antibodies were added to the cells and the samples were incubated for 40 minutes at 4°C. APC-conjugated and AF488-conjugated mouse IgG isotype antibodies were used as controls. Stained cells were washed with 1× PBS and analyzed on a Becton Dickinson FACSort instrument equipped with CellQuest software (BD Biosciences).

In vitro cytotoxicity assay

The cytotoxicity of the immunotoxins on cultured cell lines (NR6W, NR6M, and A431) and cells freshly isolated from glioblastoma xenografts (43, D08-0493MG, D270MG, and D2159MG) was assayed by inhibition of protein synthesis as described in an earlier study (32). The cytotoxic activity was defined by IC_{50}, which was the toxin concentration that suppressed incorporation of radioactivity by 50% as compared with the radioactivity measured in cells that were not treated with toxin.

In vivo intracranial tumor model and convection-enhanced delivery

Male NOD scid gamma (NSG) mice (∼30 g; 8–12 weeks, Duke University, Division of Laboratory Animal Resources) were anesthetized by an intraperitoneal injection of a solution of ketamine 50 mg/kg (Fort Dodge Animal Health) and xylazine 10 mg/kg (Akorn, Inc). The anterior cranial region was shaved under sterile conditions and an incision approximately 1 cm in length was made in the skin over the skull, and a small burr hole was drilled at coordinates 2.5 mm left lateral of the sagittal and 1 mm anterior to the bregma. A 25-gauge 10-µL Hamilton needle was inserted vertically to a depth of 2.5 mm from the dura mater. All mice were implanted with 10^5 43 or D270MG xenograft cells in 3 µL of 1× PBS or NR6M cells in 3 µL of BD PuraMatrix peptide hydrogel (BD Biosciences) into the cortex.

Six (43 xenograft), 5 (NR6M), or 11 (D270MG) days after tumor implantation, animals were randomized into 4 groups (sham $n = 4/5$, others $n = 8–10$), Sham (no pump), 0.2% PBS–HSA, P588-(scdsFv)-PE38KDEL, and D2C7-(scdsFv)-PE38KDEL. One microgram of D2C7-(scdsFv)-PE38KDEL or P588-(scdsFv)-PE38KDEL diluted in 100 µL of 0.2% PBS–HSA was delivered via alzet osmotic mini pumps (Durect Corporation) over a 3-day (43 xenograft) or 7-day (NR6M and D270MG) period. The mice were followed to assess tumor development and death.

Evaluation of intracranial xenograft response to treatment

The response of intracranial xenografts to treatment was assessed by percentage increase in time to a specific neurologic endpoint (seizure activity, repetitive circling, or other subtle changes such as grooming or decrease in appetite) or to death. Statistical analysis was conducted using the Wilcoxon rank order test as previously described (38, 39). Animals were observed twice daily for signs of distress or development of neurologic symptoms, at which time, the mice were euthanized.

Results

Determination of D2C7 epitope on EGFRvIII/EGFRwt

As several EGFR deletion mutants have been described in gliomas (19), determining the epitope of D2C7 on EGFRwt/EGFRvIII will aid in the identification of specific EGFR mutations that could be therapeutically targeted with the D2C7 mAb. Accordingly, several deletion mutants of human EGFRvIII extracellular domain (ECD) were generated. The different EGFRvIII ECD proteins were expressed as Nus-Tag fusion proteins and purified as described in Supplementary Materials. D2C7 recognized three types of C-terminal (CT) protein constructs (CT 179–356 amino acids, CT 267–356 amino acids, and CT 287–356 amino acids) but not the N-terminal (NT) protein construct (NT 1–178 amino acids) in ELISA (data not shown). Further deletions of the EGFRvIII ECD peptide fragment CT 287–356 amino acids showed strong reactivity of D2C7 to CT 292–346

amino acids and a weak reactivity to CT 295–345 amino acids (Fig. 1). These results indicated that the minimum epitope of D2C7 is 51 amino acids (CT 295–345), but 55 amino acids (CT 292–346) are essential for strong binding by D2C7 (Fig. 1 Inset).

Cloning of the V_H and V_L domain of D2C7 IgG1κ

V_H and V_L cDNAs were isolated from the D2C7 hybridoma as described in Supplementary Materials. The V_H and V_L domains were cloned and sequenced, and the fragments were 360 and 321 bp, respectively. The deduced amino acid sequences of the D2C7 V_H and V_L domains are shown in Supplementary Fig. S1. Sequence analysis of the V_H and V_L amino acids, using the NIH database of germline genes (http://www.ncbi.nlm.nih.gov/igblast/), revealed that the sequences were derived from different germ-line V genes, with a similarity of 70% to 75%.

Construction, expression, and purification of D2C7-(scdsFv)-PE38KDEL immunotoxin

The C-terminus of the D2C7 V_H domain was connected to the amino terminus of the V_L domain by the 15-amino-acid peptide $(Gly_4Ser)_3$ linker. To obtain a stable immunotoxin, it is essential to ensure that, during renaturation, V_H is positioned near V_L. This was achieved by mutating a single-key residue in each chain to cysteine for the stabilizing disulfide bond to form. Following procedures used for developing other dsFv-recombinant immunotoxins, we chose to mutate residue 44 in the framework region 2 (FR2)
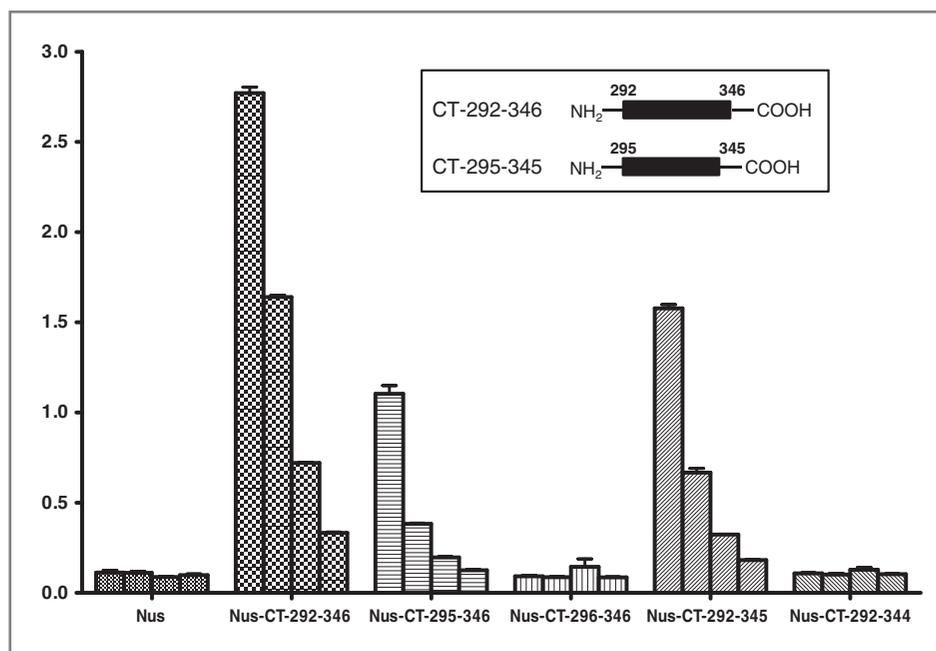

Figure 1. Determination of the epitope of D2C7 on EGFRvIII ECD. Different concentrations (0.5, 0.25, 0.125, and 0.0625 μg) of Nus-Tag alone or Nus-Tag conjugated to EGFRvIII ECD deletion mutants (CT 292-346, CT 295-346, CT 296-346, CT 292-345, and CT 292-344) were immobilized on 96-well plates. The wells were blocked with superblock and incubated with D2C7 mAb followed by peroxidase conjugated anti-mouse IgG antibody. Following the addition of TMB substrate solution, and stop solution, the optical density was measured at 450 nm. The ELISA assay with different concentrations of Nus-Tag alone or Nus-Tag conjugated to EGFRvIII ECD deletion mutants was conducted in triplicate for each epitope. Results are presented as mean ± SD where $n > 2$. Inset represents the EGFRvIII ECD peptides essential for minimum (CT 295-345) and strong binding (CT 292-346) of D2C7.

of V_H and residue 100 in the FR4 of V_L (according to the Kabat numbering) to cysteine (40). Thus, we prepared an Fv containing both a peptide linker and a disulfide bond generated by cysteine residues that replace Ser44 of V_H and Gly100 of V_L. The D2C7-(scdsFv) PCR fragment was then fused to DNA for domains II and III of *Pseudomonas* exotoxin A. The *Pseudomonas* exotoxin A version used here, PE38KDEL, has a modified C-terminus that increases its intracellular retention, thereby enhancing its cytotoxicity. The D2C7-(scdsFv)-PE38KDEL was expressed and purified as described in Materials and Methods to more than 95% purity (Supplementary Fig. S2).

Antigen-binding characteristics of D2C7-(scdsFv)-PE38KDEL antibody

The antigen-binding capability of the D2C7-(scdsFv)-PE38KDEL immunotoxin was assessed by surface plasmon resonance (Biacore, GE Healthcare). The purified D2C7-(scdsFv)-PE38KDEL was applied to sensor chips coated with either purified recombinant EGFRwt or EGFRvIII ECD proteins (Supplementary Fig. S3). The D2C7-(scdsFv)-PE38KDEL bound to both EGFRwt- and the EGFRvIII-ECD–protein-coated chips. The K_D of D2C7-(scdsFv)-PE38KDEL on the EGFRwt- and EGFRvIII-coated chips were 1.6×10^{-9} and 1.3×10^{-9} mol/L, respectively. Thus, the cloned D2C7-(scdsFv)-PE38KDEL immunotoxin bound with similar kinetics to both the wild-type and the mutant EGFR proteins.

To determine whether the D2C7-(scdsFv)-PE38KDEL immunotoxin binds to native EGFRwt and EGFRvIII proteins expressed on the cell surface, indirect flow cytometric analysis was conducted (Fig. 2). FACS analysis revealed that the D2C7-(scdsFv)-PE38KDEL bound to both the EGFRwt-expressing NR6W cells (Fig. 2B) and the EGFRvIII-expressing NR6M cells (Fig. 2C). The parental NR6 cells (Fig. 2A) and the nonspecific immunotoxin p588-(scdsFv)-PE38KDEL were used as negative controls, which confirmed the binding specificity of D2C7-(scdsFv)-PE38KDEL. These results show that the D2C7-(scdsFv)-PE38KDEL immunotoxin binds to both purified EGFRwt and EGFRvIII proteins on a chip and to native protein molecules expressed on transfected cells.

Cytotoxicity of D2C7-(scdsFv)-PE38KDEL on transfected cells and cancer cells

We next examined the effects of the D2C7-(scdsFv)-PE38KDEL immunotoxin on EGFRwt- or EGFRvIII-transfected NR6W and NR6M cell lines, respectively. The ability of the D2C7-(scdsFv)-PE38KDEL to inhibit protein synthesis was used as a measure of its cytotoxic effect. The cytotoxicity of the D2C7-(scdsFv)-PE38KDEL immunotoxin was compared with that of a known EGFRwt-specific immunotoxin, TP-38 (30), and that of a known EGFRvIII-specific immunotoxin, MR1-1 (32). We initially evaluated the cytotoxicity of the various immunotoxins to the EGFRwt-expressing NR6W cells. The IC$_{50}$ of the D2C7-(scdsFv)-PE38KDEL immunotoxin on NR6W cells was 10 times lower than that of the EGFRwt-specific immunotoxin, TP-38 (0.467 vs.
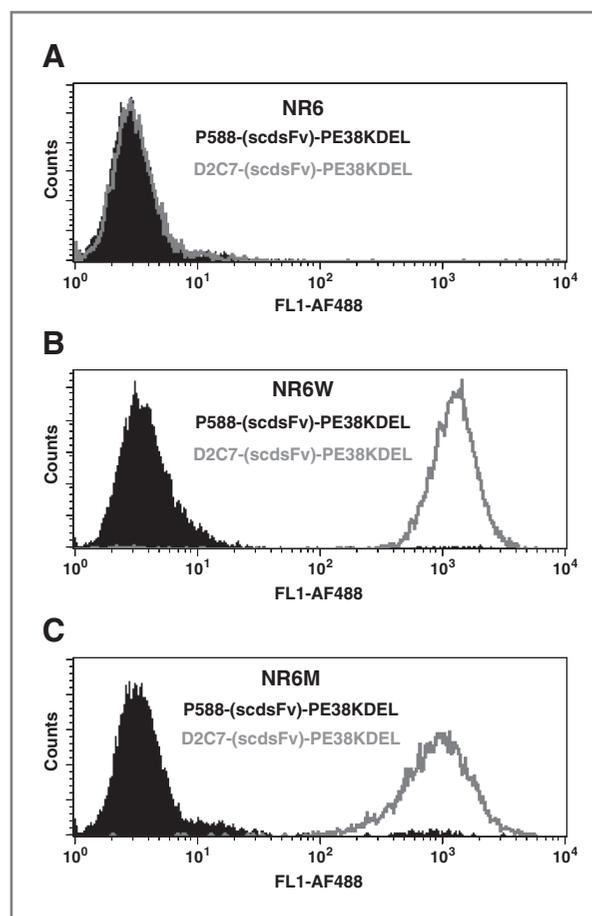

Figure 2. Flow cytometric analysis of D2C7-(scdsFv)-PE38KDEL to determine reactivity of the D2C7 immunotoxin. A, Parental NR6 cells were used as control. Indirect FACS analysis shows the reactivity of D2C7-(scdsFv)-PE38KDEL immunotoxin with cells expressing EGFRwt (NR6W) (B) or EGFRvIII (NR6M) (C). Cells were stained with D2C7-(scdsFv)-PE38KDEL (gray open peaks) or a nonspecific scFv–derived immunotoxin [P588-(scdsFv)-PE38KDEL] control (filled black peaks).

4.6; Table 1). Even on the NR6M cells, D2C7-(scdsFv)-PE38KDEL immunotoxin had an IC$_{50}$ value 1.6 times lower than that of the EGFRvIII-specific immunotoxin, MR1-1 (0.253 vs. 0.413; Table 1).

The cytotoxic effects of D2C7-(scdsFv)-PE38KDEL were also tested on various EGFRwt- and EGFRvIII-positive human cancer cells. The A431-epidermoid-carcinoma cell line overexpresses wild-type EGFR protein. Freshly isolated cells from glioblastoma xenografts, 43 and D08-0493MG express EGFRwt protein, and D2159MG and D270MG express both EGFRwt and EGFRvIII proteins. Prominently, FACS analysis showed the coexpression of cancer stem cell marker CD133 and D2C7 on 71% and 86% of cells from glioblastoma xenografts 43 and D2159MG, respectively (Supplementary Fig. S4). As shown in Table 2, the D2C7-(scdsFv)-PE38KDEL was highly effective, with an IC$_{50}$ of 0.18 to 2.5 ng/mL, in killing the A431 human cancer cell line and all of the glioblastoma xenograft cells tested.

Table 1. Cytotoxicity of D2C7 (EGFRwt and EGFRvIII), TP-38 (EGFRwt), and MR1-1 (EGFRvIII) immunotoxins toward EGFRwt/EGFRvIII-transfected cell lines[a]

Cell line	D2C7 IC$_{50}$ ng/mL	TP-38 IC$_{50}$ ng/mL	MR1-1 IC$_{50}$ ng/mL
NR6W	0.467 ± 0.18	4.6 ± 2.2	74.7 ± 17.9
NR6M	0.253 ± 0.04	101.7 ± 14.4	0.413 ± 0.03

[a]Cytotoxicity data are given as an IC$_{50}$ value, the concentration of immunotoxin that causes a 50% inhibition of protein synthesis after a 20-hour incubation with immunotoxin. All the assays were conducted in triplicate for each cell line. Results are presented as mean ± SD where $n \geq 2$.

Stability of D2C7-(scdsFv)-PE38KDEL immunotoxin

The therapeutic efficacy of an immunotoxin is greatly influenced by its stability. Hence, the stability of D2C7-(scdsFv)-PE38KDEL was determined at two different concentrations over a 7-day period at 37°C by measuring its cytotoxic activity against NR6M cells as described in Supplementary Materials. Statistical analysis ($P = 0.103$) of the 7-day IC$_{50}$ values established D2C7-(scdsFv)-PE38KDEL to be highly stable throughout the assay period (Supplementary Fig. S5).

Efficacy of D2C7-(scdsFv)-PE38KDEL in intracranial tumor models

The D2C7-(scdsFv)-PE38KDEL is human specific and does not react with murine EGFR (Supplementary Fig.

Table 2. Cytotoxicity of D2C7 immunotoxin toward various cancer cells[a]

Cell line	Cancer type	D2C7-(scdsFv)-PE38KDEL IC$_{50}$ ng/mL
A431 (EGFRwt)	Epidermoid carcinoma	0.180 ± 0.014
43 (EGFRwt)	Glioblastoma	2.28 ± 0.85
D08-0493MG (EGFRwt)	Glioblastoma	2.5 ± 0.99
D2159MG (EGFRwt and EGFRvIII)	Glioblastoma	0.204 ± 0.220
D270MG (EGFRwt and EGFRvIII)	Glioblastoma	0.265 ± 0.134

[a]All the cells are of human origin. Cytotoxicity data are given as an IC$_{50}$ value; the concentration of immunotoxin that causes a 50% inhibition of protein synthesis after a 20-hour incubation with immunotoxin. All the assays were conducted in triplicate for each cell line. Results are presented as mean ± SD where $n \geq 2$.
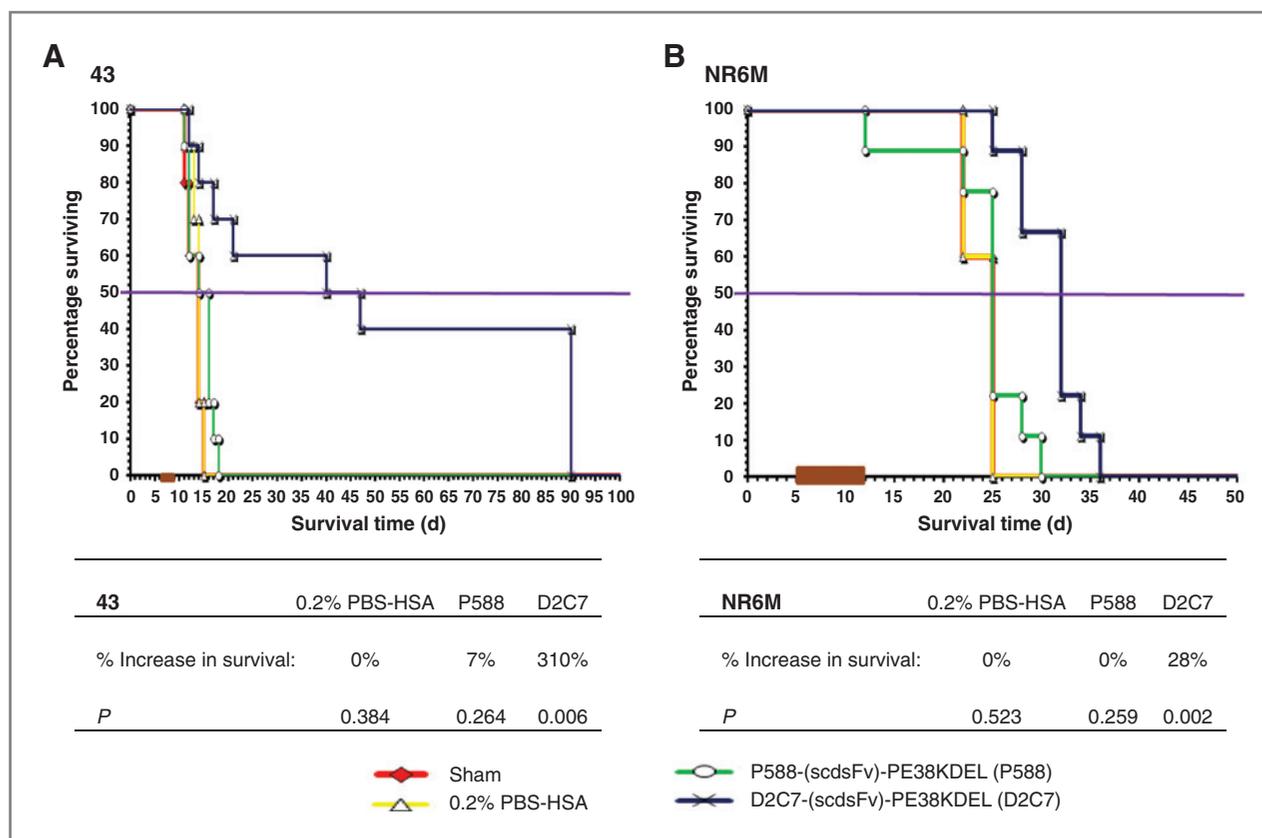
S6). As a model for tumors expressing EGFRwt, the glioblastoma xenograft 43 was chosen; as a model for tumors expressing both EGFRwt and EGFRvIII, the glioblastoma xenograft D270MG was chosen. As the *EGFRvIII* mutation is most frequently reported in glioblastomas with *EGFR* amplification (19), there is no true glioblastoma xenograft that expresses *EGFRvIII* only. Therefore, we selected the fibroblast cell line NR6M, transfected with the human *EGFRvIII* as a model for EGFRvIII-expressing tumor. The survival curve graph for 43 (Supplementary Fig. S7a), NR6M (Supplementary Fig. S7b), and D270MG (Supplementary Fig. S7c) showed that 100% death occurred at day 18, day 15, and day 36 post tumor implantation for 43, NR6M, and D270MG, respectively. Therefore, day 6 (43 xenograft), day 5 (NR6M), and day 11 (D270MG) post tumor implantation were chosen as the optimal days to study the efficacy of D2C7-(scdsFv)-PE38KDEL. Furthermore, toxicity studies in NSG mice with different concentrations of the D2C7-(scdsFv)-PE38KDEL (1–10 μg/100 μL) showed that 1 μg of D2C7-(scdsFv)-PE38KDEL had no toxicity-associated mortality (Supplementary Fig. S8).

In the 43 intracranial tumor model overexpressing EGFRwt protein in the absence of *EGFRwt* amplification, orthotopic delivery of D2C7-(scdsFv)-PE38KDEL prolonged the survival by 310% ($P = 0.006$; Fig. 3A). The group treated with the control immunotoxin, P588-(scdsFv)-PE38KDEL, showed only a 7% increase in survival with a P value of 0.264. Four of ten 43 tumor-bearing mice in the D2C7-(scdsFv)-PE38KDEL treatment group were still alive at the termination of the study. Similarly, in the EGFRvIII-expressing NR6M orthotopic tumor model, the control immunotoxin, P588-(scdsFv)-PE38KDEL, failed to show any response whereas D2C7-(scdsFv)-PE38KDEL treatment showed a statistically significant ($P = 0.002$) increase in survival by 28% (Fig. 3B). Likewise, in the D270MG intracranial tumor model expressing both EGFRwt and EGFRvIII, delivery of D2C7-(scdsFv)-PE38KDEL by CED prolonged the survival by 166% ($P = 0.001$; Fig. 4). Notably, 8 of 10 of the D270MG tumor-bearing mice in the D2C7-(scdsFv)-PE38KDEL treatment group were still healthy and alive at the termination of the study.

Assessment of D2C7-(scdsFv)-PE38KDEL tumor distribution after CED

Sufficiently high concentrations of D2C7-(scdsFv)-PE38KDEL in the tumor area are achieved by CED. However, the antitumor efficacy of D2C7-(scdsFv)-PE38KDEL depends on its homogenous distribution within the tumor area. Tumor distribution of D2C7-(scdsFv)-PE38KDEL administered by CED in D270MG intracranial model was studied by immunohistochemistry. Tumor sections from the D270MG-sham group and the D270MG-D2C7-(scdsFv)-PE38KDEL group were used as the negative control (Supplementary Fig. S9A) and the test specimen (Supplementary Fig. S9C), respectively. The D270MG-D2C7-(scdsFv)-PE38KDEL group, pre-stained with D2C7-

Figure 3. Effect of D2C7-(scdsFv)-PE38KDEL on 43 and NR6M intracranial tumors in NSG mice. Male NSG mice (~30 g; 8–12 weeks) bearing 43 (A) or NR6M (B) tumors were randomized into four groups: Sham, 0.2% PBS/HSA, P588-(scdsFv)-PE38KDEL, and D2C7-(scdsFv)-PE38KDEL. The test mice were treated over a 3 (43 xenograft) or 7 (NR6M) -day period with a total of 1 µg of D2C7-(scdsFv)-PE38KDEL diluted in 100 µL 0.2% PBS-HSA. Control mice were either left untreated (Sham) or treated with 0.2% PBS–HSA or 1 µg of the control immunotoxin, P588-(scdsFv)-PE38KDEL, diluted in 100 µL 0.2% PBS–HSA.

(scdsFv)-PE38KDEL, served as the positive control (Supplementary Fig. S9B). Supplementary Figs. S9B and S9C clearly show the identical staining pattern of both the positive control and the test specimen, thereby establishing the uniform distribution of D2C7-(scdsFv)-PE38KDEL within the tumor area.

Discussion

To achieve the clinical goal of successful immunotherapy for solid intracranial tumors, investigators have begun developing mAbs or recombinant scFvs against tumor antigens. One such antibody, D2C7, recognizes both the EGFRwt and the mutant EGFRvIII, two proteins that are overexpressed in glioblastoma. The D2C7 antibody exhibited significant reactivity against both EGFRwt and EGFRvIII on tissue sections from adult patients with glioblastoma. It also showed significant affinity by surface plasmon resonance and specificity by flow cytometry (36). The high affinity, specificity, and reactivity of this mAb make it an ideal candidate for the construction of a recombinant immunotoxin that targets tumors overexpressing EGFRwt and EGFRvIII proteins. Due to the prevalence of different EGFR deletion mutants in glioblastoma, we deemed it necessary to identify the epitope for D2C7 on its target

EGFRwt/EGFRvIII proteins. The 55 amino acid D2C7 epitope (EGFRwt 583–637 amino acids) is known to be present in EGFR deletion mutants C-958, Δ959–1030, Δ6–185, I, III–VII (19, 41), thereby increasing the number of antigenic targets for D2C7.

Subsequently, we cloned an EGFRwt/EGFRvIII-specific immunotoxin, D2C7-(scdsFv)-PE38KDEL, from the D2C7 hybridoma and characterized its efficacy using *in vitro* and *in vivo* models. The *in vitro* cytotoxicity data showed that D2C7-(scdsFv)-PE38KDEL effectively inhibits protein synthesis in a variety of EGFRwt- or EGFRwt–EGFRvIII-expressing glioblastoma xenograft cells and human tumor cell line. Notably, in the intracranial animal models of EGFRwt-expressing glioma xenograft 43, EGFRvIII-expressing NR6M, and EGFRwt- and EGFRvIII-expressing glioblastoma xenograft D270MG, D2C7-(scdsFv)-PE38KDEL showed significant increase in survival, 310% ($P = 0.006$), 28% ($P = 0.002$), and 166% ($P = 0.001$), respectively. To the best of our knowledge, this is the first report showing that an immunotoxin can target both the wild-type EGFR and the mutant EGFRvIII in glioma models.

To show significant therapeutic efficacy when administered to patients with brain tumors, an immunotoxin has to be considerably stable over long periods of time.
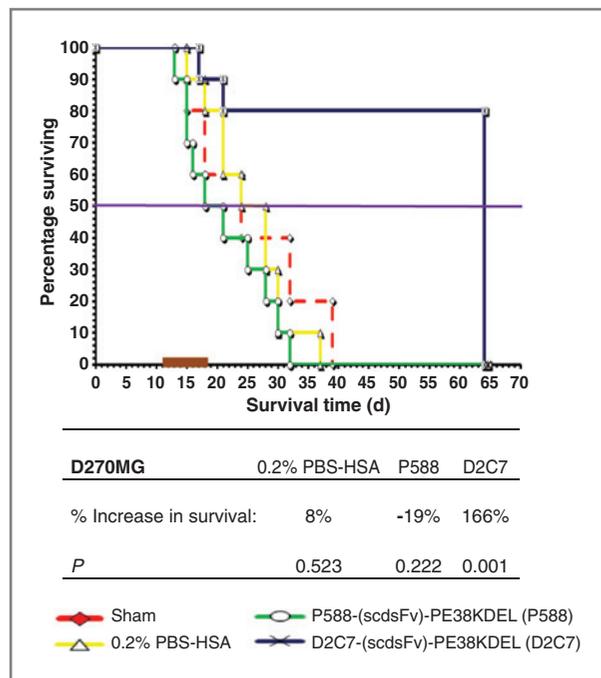
Figure 4. Effect of D2C7-(scdsFv)-PE38KDEL on D270MG intracranial tumors in NSG mice. Male NSG mice (~30 g; 8–12 weeks) bearing D270MG tumors were randomized into four groups: Sham, 0.2% PBS/HSA, P588-(scdsFv)-PE38KDEL, and D2C7-(scdsFv)-PE38KDEL. The test mice were treated over a 7-day period with a total of 1 μg of D2C7-(scdsFv)-PE38KDEL diluted in 100 μL 0.2% PBS–HSA. Control mice were either left untreated (Sham) or treated with 0.2% PBS–HSA or 1 μg of the control immunotoxin, P588-(scdsFv)-PE38KDEL, diluted in 100 μL 0.2% PBS–HSA.

Therefore, in addition to the conventional 15-amino-acid peptide linker present between the V_H and V_L domain of D2C7 scFv, we introduced an inter-chain disulfide bond between the structurally conserved framework regions of the D2C7 V_H and V_L domains, to construct a new disulfide-stabilized immunotoxin termed D2C7-(scdsFv)-PE38KDEL. The disulfide-stabilized D2C7-(scdsFv)-PE38KDEL was highly stable over a 7-day period, with a P value of 0.103.

In in vitro studies, D2C7-(scdsFv)-PE38KDEL inhibited protein synthesis in EGFRvIII-expressing NR6M cells at a concentration 402 times lower than TP-38, an EGFRwt-specific TGF-α–based immunotoxin. Furthermore, D2C7-(scdsFv)-PE38KDEL inhibited protein synthesis at a concentration 160 times lower than MR1-1, an EGFRvIII-specific immunotoxin, in EGFRwt-expressing NR6W cells. Any therapy that fails to target the EGFRwt-expressing cells or constitutively autophosphorylated EGFRvIII-expressing cells will lead to enhanced proliferation and migration of these tumor cells. Furthermore, any surviving EGFRvIII-only–expressing tumor cells in tumors that have both EGFRwt and EGFRvIII will promote resistance to EGFR antibody therapy (23). In our in vitro studies with EGFRwt- or both EGFRwt- and EGFRvIII-expressing human cancer cells, D2C7-(scdsFv)-PE38KDEL showed significant inhibition of protein synthesis. Furthermore, the D2C7-(scdsFv)-

PE38KDEL immunotoxin prolonged survival in in vivo tumor models with the 43 xenograft, NR6M, and D270MG xenograft. Examination of brain sections from the 8 euthanized mice from the D270MG-D2C7-(scdsFv)-PE38KDEL treatment group by hematoxylin and eosin staining revealed no tumor cells in 8 of 8 mice. Macrophage or monocyte infiltration was observed in 4 of 8 mice. No expression of EGFRwt (EGFR1 mAb), EGFRvIII (L8A4 mAb), or EGFRwt and EGFRvIII (D2C7 mAb) was observed in 8 of 8 mice, indicating that all the 8 mice in the D270MG-D2C7-(scdsFv)-PE38KDEL treatment group were cured. Therefore, we believe that D2C7-(scdsFv)-PE38KDEL is likely to be more efficacious in treating patients of glioblastoma whose tumors express both EGFRwt and EGFRvIII or either EGFRwt or EGFRvIII alone. Furthermore, EGFR mutants form homo/heterodimers and currently available EGFR mAbs cetuximab, matuzumab, and panitumumab are unable to block activation of these dimers (42). As the D2C7 epitope is present on a wide variety of these EGFR deletion mutants, D2C7-(scdsFv)-PE38KDEL treatment should prove efficacious for patients with glioblastoma harboring these mutations.

For effective in vivo therapy of glioblastoma, it is essential to achieve high local concentration and homogeneous distribution of the immunotoxin at the tumor site. In the 43, NR6M, and D270MG models, D2C7-(scdsFv)-PE38KDEL was administered by continuous intracranial delivery through osmotic mini-pumps. This method of continuous intracranial delivery will aid in achieving elevated concentrations and uniform distribution of D2C7-(scdsFv)-PE38KDEL at the tumor site, which would be expected to optimize its antitumor activity. By this method, we were able to achieve significant increase in survival at a very low dose of 1 μg of D2C7-(scdsFv)-PE38KDEL.

D2C7-(scdsFv)-PE38KDEL might cause human skin and liver toxicity if the immunotoxin were administered by systemic injection. However, as we propose to administer the immunotoxin by CED directly into intracranial tumors, any systemic toxicity due to low and clinically insignificant amounts of immunotoxin gaining access to systemic organs and tissues would be minimized by this localized treatment. The regional drug delivery technique of CED has been used successfully to deliver recombinant toxins targeting EGFRwt (30) and EGFRvIII (43) in patients with malignant glioma, without any liver or other systemic organ toxicity.

The in vitro internalization studies showed rapid uptake of D2C7-(scdsFv)-PE38KDEL by NR6W and NR6M cells within 1 to 2 hours of treatment (data not shown). We hypothesize a similar uptake of D2C7-(scdsFv)-PE38KDEL by tumor cells upon infusion and fast clearance of the immunotoxin within the tumor area. Moreover, our immunohistochemical studies in the D270MG orthotopic model clearly show that homogenous distribution of the D2C7-(scdsFv)-PE38KDEL is essential in generating a significant antitumor response. Consequently, tumor cells that failed to encounter D2C7-(scdsFv)-PE38KDEL might regrow and repopulate the tumor area. Thus, tumor recurrence in our in vivo models might be due to both the swift depletion of the

low levels of D2C7-(scdsFv)-PE38KDEL (1 μg) and lack of uniform distribution, and not due to escape from immunotoxin efficacy.

Because of the recurrent nature of malignant gliomas, as well as the diversity of antigens populating the glioma cell surface, there is a need for innovative immunotherapeutic approaches. In an earlier study, Schmidt and colleagues described a recombinant immunotoxin, scFv(14E1)-ETA, which bound both EGFRwt and EGFRvIII (34). The activity of scFv(14E1)-ETA was shown both *in vitro* and *in vivo*, but only on the epidermoid carcinoma cell line A431 or the EGFRwt- or EGFRvIII-transfected murine renal carcinoma cells (Renca-lacZ/EGFR or Renca-lacZ/EGFRvIII) and not on EGFRwt- and EGFRvIII-expressing human glioma models. Moreover, the study by Schmidt and colleagues did not include immunohistochemical analyses that would show the binding of their construct to human glioma tissue or any other human cancer tissue. In this regard, D2C7-(scdsFv)-PE38KDEL is a novel immunotoxin that can target both the EGFRwt and the EGFRvIII proteins that are frequently overexpressed in malignant gliomas.

Monoclonal antibodies 528 and 806, with dual specificity for wild-type and mutant EGFR proteins expressed on different cell lines, have been well described in previous studies (44, 45). Combination therapy using mAbs 528 and 806 showed a significant decrease in tumor volume of xenografts expressing EGFRwt or EGFRvIII (46). Such combination therapies targeting multiple tumor-associated molecules involve high development, manufacturing, and treatment costs. These intrinsic difficulties associated with combination antibody therapies will be circumvented with our EGFRwt/EGFRvIII-specific D2C7-(scdsFv)-PE38KDEL.

In conclusion, we have created a scFv molecule that is capable of mediating selective *in vitro* and *in vivo* tumor targeting. We believe this to be the first significant evidence showing enhanced glioblastoma tumor targeting with high selectivity and specificity by an antibody specific for two glioma-associated antigens. Taken together, our results suggest that D2C7-(scdsFv)-PE38KDEL should be clinically efficacious against brain tumors expressing EGFRwt or EGFRvIII alone or together. A GLP clinical preparation of D2C7-(scdsFv)-PE38KDEL has been prepared, after which we will obtain a U.S. Food and Drug Administration Investigational New Drug (FDA IND). NCI funding (P01 CA154291-01) for phase I and II trials of D2C7-(scdsFv)-PE38KDEL by CED in patients with glioblastoma has been obtained. A previous, randomized trial of an IL-13-PE38 immunotoxin in recurrent patients with glioblastoma failed to show a survival advantage over Gliadel wafers (47). There was no imaging of delivery in that trial, and later studies

showed that the IL-13 receptor is only expressed in approximately 40% of glioblastomas (48). Moreover, there was heterogeneous expression among glioblastoma cells within 40% of positive cases. It is likely that the IL-13-PE38 trial failed because of poor delivery from the lack of imaging during delivery, as well as the low, heterogeneous expression of the IL-13 receptor on a minority of glioblastoma cases.

We have recently shown successful imaging of an immunotoxin, MR1-1-PE38, similar in size to our D2C7-(scdsFv)-PE38KDEL, in a clinical trial with CED. Successful imaging of the delivery of D2C7-(scdsFv)-PE38KDEL will greatly improve treatment efficacy (43, 49). As discussed earlier, the high levels of homogeneous expression of the D2C7-(scdsFv)-PE38KDEL molecular targets, wild-type EGFR and EGFRvIII (both of which are major glioblastoma driver oncogenes), in more than 95% of newly diagnosed glioblastoma tumors will also improve D2C7-(scdsFv)-PE38KDEL efficacy over other immunotoxins previously used in glioblastoma clinical trials.

Authors' Contributions

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Construction of an Immunotoxin, D2C7-(scdsFv)-PE38KDEL, Targeting EGFRwt and EGFRvIII for Brain Tumor Therapy

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