A bispecific immunotoxin (DTAT13) targeting human IL-13 receptor (IL-13R) and urokinase-type plasminogen activator receptor (uPAR) in a mouse xenograft model

Deborah A. Todhunter1, Walter A. Hall1, Edward Rustamzadeh1, Yanqun Shu2, Sekou O. Doumbia2 and Daniel A. Vallera2,3

1Department of Neurosurgery and 2Department of Therapeutic Radiology–Radiation Oncology, Section on Experimental Cancer Immunology, University of Minnesota Cancer Center, Mayo Mail Code 367, Harvard St at East River Road, Minneapolis, MN 55455, USA
2To whom correspondence should be addressed.
E-mail: valle001@tc.umn.edu

A bispecific immunotoxin (IT) called DTAT13 was synthesized in order to target simultaneously the urokinase-type plasminogen activator receptor (uPAR)-expressing tumor neovasculature and IL-13 receptor expressing glioblastoma cells with the goal of intratumoral administration for brain tumors. The recombinant hybrid was created using the non-internalizing N-terminal fragment (ATF) of uPA and the IL-13 molecule for binding plus the catalytic and translocation portion of diphtheria toxin (DT) for killing. The 71 kDa protein was highly selective for human glioblastoma in vitro showing no loss on binding compared with DTAT and DTIL13 controls. In vivo, DTAT13 caused the regression of small tumors when administered at 10 μg/day given on a five-dose schedule every other day. DTAT13 was able to target both overexpressed uPAR and the vasculature, as demonstrated by its ability to kill HUVEC cells. Also, mortality studies indicated that DTAT13 was less toxic than DTAT or DTIL13. These findings indicate that bispecific IT may allow treatment of a broader subset of antigenically diverse patients while simultaneously reducing the exposure to toxin required than if two separate agents were employed.

Keywords: diphtheria toxin/glioblastoma multiforme/immunotoxin/interleukin-13/urokinase-type plasminogen activator

Introduction

Glioblastoma multiforme (GBM), the most common primary brain tumor, is highly invasive, diffuse, well vascularized and morphologically heterogeneous. Surgery, chemotherapy and radiotherapy do little to change the poor survival of people diagnosed with GBM (Davis et al., 1998; Gurney and Kadan-Lottick, 2001). Since the traditional treatments are not effective, other methods involving targeted specific markers on GBM are under investigation (Debinski, 2002; Mischel and Cloughesy, 2003). One approach that shows remarkable potential is selectively targeting catalytic toxins such as diphtheria toxin (DT) with tumor-specific ligands.

A logical ligand choice for making these immunotoxins (IT) is IL-13R, since it is overexpressed on GBM (Debinski et al., 1995a, 1999) and minimally expressed on normal cells. The IL-13 receptor (IL13R) on GBM is interleukin-4 (IL-4) independent, different from the IL13R on normal tissue (Debinski et al., 1998) and termed IL13Rα2 (Debinski et al., 1999). Normal brain tissue and other organs and tissues, with the exception of testes, express little or no IL13Rα2 (Debinski and Gibo, 2000; Joshi et al., 2000). The IL13R on GBM has been used as a target for IT consisting of human IL-13 and bacterial toxins (Debinski et al., 1995a,b; Nash et al., 2001; Li et al., 2002). All of these studies have demonstrated the versatility of IL-13 IT in its ability to kill IL13R-positive GBM in vitro (Debinski et al., 1995a,b, 1998; Husain et al., 2001; Li et al., 2002) and in vivo xenografts in mice (Debinski et al., 1999; Kawakami et al., 2002; Li et al., 2002).

A different and unrelated ligand for targeting GBM is urokinase-type plasminogen activator (uPA). Studies show the amount of urokinase-type plasminogen activator receptor (uPAR) is elevated in GBM compared with normal brain tissue (Yamamoto et al., 1994) and uPAR expression is greater in high-grade gliomas compared with low-grade gliomas (Bhattacharya et al., 2001). The importance of uPAR for glioblastoma invasion has been demonstrated in vitro by inhibition of a glioma cell line invasion by anti-uPAR monoclonal antibody (Mori et al., 2000) and by stably transfecting a GBM line with antisense uPAR mRNA (Mohanam et al., 1997). Glioma cells transfected with antisense uPAR failed to form intracerebral tumors in nude mice (Go et al., 1997). Immunotoxins targeting the uPAR coupled to saporin (Fabbri et al., 1997), Pseudomonas exotoxin (Rajagopal and Kreitman, 2000) and diphtheria toxin (Vallera et al., 2002) were cytotoxic to uPAR-expressing glioblastoma cells.

Previous studies in our laboratory and others (Debinski et al., 1995b, 1998; Joshi et al., 2000; Li et al., 2002) have shown that no single IT appears to be inclusive in recognizing all GBM. Initial studies with a truncated form of DT (DT390) fused to human IL-13 (DTIL13) showed that although some GBM were highly susceptible to the toxic effects of DTIL13, other cell lines were not (Li et al., 2002). A second IT (Vallera et al., 2002), truncated DT390 fused to the N-terminal fragment (ATF) of uPA and called DTAT, was synthesized as an alternative IT for GBM that were insensitive to the cytotoxic effects of DTIL13. In addition to targeting GBM, DTAT also targeted the tumor vasculature (Vallera et al., 2002). The truncated DT for both proteins excluded the native receptor binding domain and consisted of the ADP ribosylating domain and the hydrophobic membrane-translocating region of the molecule (Li et al., 2002; Vallera et al., 2002). Diphtheria toxin was chosen because one molecule of DT in the cell cytosol can kill a cell (Yamaizumi et al., 1978). Some of the problems and dose-limiting side effects of a bacterial toxin-based IT are antigenicity of the bacterial portion of the molecule and damage to liver, kidney and blood vessel endothelium (Foss et al., 1998).
Based on our previous findings that DTAT was effective against glioblastoma tumors in vivo (Vallera et al., 2002), in this study we devised an alternative strategy for DTIL13-unresponsive tumors. We added the ATF of uPA to the DTIL13 molecule in such a way as to retain site-specific binding of both ligand components. The purpose of this molecular alteration was (1) to broaden the reactivity against the maximum number of glioblastoma cases, (2) to target the vasculature and (3) to reduce toxicity since only one DT molecule would be present on each DTAT13 molecule. Despite combining both ligands on the same toxin molecule, DTAT13 retained its anti-vasculature effect and its anti-glioblastoma efficacy. Also, it appeared to have less toxic side effects than DTAT and DTIL13.

Materials and methods

Production of DTAT13

The DTAT13 gene was assembled as described previously (Vallera et al., 2002). The hybrid gene consisted of the first 389 amino acids of diphertheria toxin, an EASGGE linker, 112 amino acids of IL-13 peptide, a 15 amino acid (G4-S)3 linker and the 135 amino acid ATF from uPA. The DTAT13 fragment was subcloned into NcoI and XhoI cut pET21d expression vector (Novagen, Madison, WI) to form DTAT13/pET21d (Figure 1). Escherichia coli BL21(DE3) (Novagen, Madison, WI) cells were transformed with DTAT13/pET21d. Protein expression was induced by isopropyl-β-D-thiogalactoside and DTAT13 was isolated from inclusion bodies. Isolation from inclusion bodies, solubilization and renaturation of DTAT13 were described previously (Vallera et al., 2002). DTAT13 was purified by ion-exchange chromatography (Q Sepharose Fast Flow; Sigma, St Louis, MO) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was puriﬁed by ion-exchange chromatography (Q Sepharose Fast Flow; Sigma, St Louis, MO) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentration. Puriﬁed (Coomassie Plus Protein Assay Reagent; Pierce, Rockford, IL) and Bradford assay was used to determine protein concentra...
suture. The Hamilton syringe was mounted on the Kopf stereotaxic instrument and the needle inserted to a depth of 2.5 mm into the right cerebral hemisphere of the mouse. After injection of 5 or 10 μl of IT, the needle was slowly withdrawn. The incision was sutured and the mice recovered in a warmed cage. Subsequent IC administration of IT was performed using an identical setup and injections were made through the same burr hole.

**Histology**

Mice were killed, autopsied and tissues were taken for histopathological analysis as described (Vallera et al., 1997). Tissue samples from liver, kidney, brain, spleen and skin were collected. All samples were put in OCT embedding compound (Tissue-Tek, Electron Microscopy Sciences, Washington, PA), snap frozen in liquid nitrogen and stored at −80°C until sectioned. Serial 4 μm sections were cut, thaw mounted on glass slides and fixed for 5 min in acetone. Slides were stained with hematoxylin and eosin (H&E) for histopathological assessment.

**Statistical analysis**

Groupwise comparisons of continuous data were made by Student’s t-test.

**Results**

**Purity of DTAT13**

To determine the size and purity of the recombinant agents, DTAT13 was isolated on a Q Sepharose anion-exchange column. SDS-PAGE analysis showed that the purity of DTAT13 was >90% with a molecular weight of ~71 kDa (Figure 2). The purity and molecular weight of DTIL13 (lane 2) were 95% and 55 kDa, respectively, and for DTAT (lane 3) 90% and 58 kDa, respectively. The protein yield was 2.4, 1.25 and 0.7 mg/l bacterial culture for DTIL13, DTAT and DTAT13, respectively.

**Effect of DTAT13 on the proliferation of human glioblastoma cell lines**

To determine the anti-tumor effects of DTAT13 agents, the cytotoxic activity of DTAT13 was measured by the uptake of [³H]thymidine. Glioblastoma cell lines, U373 MG (Figure 3A) and U87 MG (Figure 3B), were incubated with increasing concentrations of DTAT13 for 72 h. Figure 3 shows that both cell lines were inhibited in a dose-dependent manner by DTAT13. The IC₅₀ of DTAT13 was 0.2 nM for U87 MG cells and 0.0007 nM for U373 MG cells. We previously demonstrated the ability of DTIL13 to kill U373 MG cells in a similar assay (Li et al., 2002). Figure 3A shows a comparison of DTAT13 with DTIL13. The IC₅₀ of DTIL13 for U373 MG cells was 0.00015 nM. Figure 3B shows the cytotoxic activity of DTAT for U87 MG cells. DTAT was more inhibitory than DTIL13 to U87 MG cells, which could be attributed to a difference in receptor numbers for IL13 and uPA. DTAT13 and DTAT have similar cytotoxic activity against U37 MG cells (Figure 3B). The IC₅₀ of DTAT and DTAT13 for U87 MG cells were 0.24 and 0.23 nM, respectively. Anti-IL13 and anti-uPA antibody were able to block the cytotoxic activity of DTAT13 (Figure 4A and B), whereas anti-murine-IL4 had no effect on DTAT13 activity. These data indicate that both the ATF molecule and IL13 moiety of DTAT13 retained their binding activity. The antibody blocking studies indicate that the anti-tumor effect of DTAT13 is mediated through both the DTIL13 and ATF portions of the molecule. In other studies, we tested the ability of DTAT13 to kill U118 MG and T98 G cells.

**Fig. 2.** Ten percent non-reduced SDS-PAGE of purified proteins, DTAT, DTIL13 and DTAT13, stained with Coomassie Brilliant Blue. Lane 1, molecular weight standards; lane 2, DTIL13; lane 3, DTAT; lane 4, DTAT13; and lane 5, immunoglobulin IgG2b. Each lane contains ~5 μg of protein. Molecular weight standards were 97.4, 66, 45 and 31 kDa.

**Fig. 3.** Effect of DTAT13 on the proliferation of human glioblastoma cell lines U373 MG and U87 MG. Cells were cultured with fusion proteins for 72 h and proliferation was measured by uptake of tritiated thymidine. Data are percentages of control response, where control response is untreated cells. Data are expressed as mean ± standard deviation (SD). (A) Activity of DTIL13 and DTAT13 against U373 MG cells. (B) Activity of DTAT and DTAT13 against U87 MG cells. Mean control values ± SD for U373 MG and U87 MG cells were 47 345 ± 6701 and 24734 ± 5092, respectively.
Glioblastoma T98 G cells were not very susceptible to the cytotoxic effects of DTAT13. Previous studies have shown that DTIL13 (Fabbrini et al., 1997) and DTAT (Mohanam et al., 1997) were ineffective at in vitro killing of T98 G cells. Cell line U118 MG responded in a dose dependent manner to the cytotoxic effects of DTAT13 with an IC50 of 0.12 nM (Figure 5). Daudi cells, a B-cell lymphoma, were unaffected by DTAT13 (Figure 5).

Cytotoxicity of DTAT13 for endothelial cells

In order to determine whether DTAT13 retained the ability to bind to the vasculature, we tested the ability of DTAT13 to kill HUVEC cells that express uPAR. DTAT has been shown to inhibit the proliferation of HUVEC cells (Vallera et al., 2002) and kill in a dose-dependent manner (Figure 6). An irrelevant control fusion protein, DT390 anti-CD3sFv made with the identical DT390 cassette (Vallera et al., 1997), did not inhibit HUVEC cells. DTAT13 killing was significant (P < 0.05). These data demonstrate that the ATF portion of the DTAT13 molecule still retains the ability to kill endothelial cells selectively by binding uPAR.

Treatment of U373 MG and U87 MG xenografts with DTAT13

Glioblastoma tumors were established via subcutaneous injection on the flank of mice by injecting 6×106 U87 MG or U373 MG cells. U373 tumors were treated with 10 μg/injection DTIL13 or DTAT13. U87 tumors were treated with 10 μg/injection of DTAT or DTAT13. All tumors were injected five times every other day and received exactly the same dosage and schedule. Treatment with either DTIL13 or DTAT13 resulted in regression of U373 MG tumors (Figure 7A). The mean tumor volume of DTIL13- and DTAT13-treated U373 MG tumors was significantly (P < 0.05) less 20–31 days post-tumor induction compared with untreated controls. Figure 7B shows the tumor volume of individual mice treated with DTIL13. All DTIL13-treated tumors regressed by 30 days. However, after 70 days, three of five tumors began to regrow. Two of five mice remained tumor free. Individual mouse data for DTAT13-treated U373 MG tumors are shown in Figure 7C. Similarly to the DTIL13-injected tumors, all DTAT13-treated
tumors regressed by day 30. At 105 days after the start of the experiment, three of the mice remained tumor free. These data indicate that DTAT13 is as effective as DTIL13 in inhibiting the U373 MG tumor.

The antitumor effects of DTAT and DTAT13 on U87 MG flank tumors in nude mice are shown in Figure 8A in a different experiment. The mean tumor volume of DTAT- or DTAT13-treated U87 MG tumors was significantly \( P < 0.05 \) less 16–26 days post-tumor induction compared with untreated controls. Examination of the data from individual mice reveals that intratumoral treatment of U87 MG tumors with DTAT or DTAT13 resulted in the rapid regression of all tumors (Figure 8B). However, tumor relapse occurred more rapidly in two of five mice on days 20–30 in the DTAT-treated group. Treatment of U87 MG tumors with DTAT13 also caused tumor regression (Figure 8C), with tumor relapse occurring in three of five between days 40 and 50 of the experiment. Two DTAT13-treated mice remained tumor free at 60 days. Taken together, Figures 7 and 8 indicate that the U373 tumors DTIL13 and DTAT13 have similar inhibitory effects. In contrast, for the U87 tumor, the results indicate that DTAT13 may be more effective than DTAT and the use of a bispecific agent may have an advantage.

Toxicity of DTAT, DTIL13 and DTAT13 in mice

To determine the toxicity of DTAT, DTIL13 and DTAT13, the effect of intratumoral injection was measured by changes in body weight. Higher doses of DTAT were used to compensate for the differences in size of the two molecules. Mice were given 24 \( \mu \)g/injection of DTAT13 and 20 \( \mu \)g/injection of DTAT so that they would receive equimolar equivalents. Despite equivalent dosages, DTAT was more toxic. Mice that were injected with 24 \( \mu \)g/injection of DTAT13 given five times did not lose weight and were similar to control mice in maintenance of body weight (Figure 9A). In contrast, mice that were injected with 20 \( \mu \)g/injection of DTAT lost weight. Mice treated with DTAT started at an average weight of ~29.8 g prior to the first injection and decreased to ~24.2 g following the course of five injections of DTAT. Injection of 10 \( \mu \)g/injection of DTAT also resulted in a slight decrease in weight compared with control or DTAT13-injected mice (Figure 9B). The body weight of mice was unaffected by injection of 10 \( \mu \)g/injection of DTIL13 or DTAT13 (Figure 9C) compared with controls.

Since our intent is to use IT for intracranial therapy, DTAT13 was injected intracranially to determine its effect on animal mortality. When injected intracranially, mice (n = 10/group) tolerated a total of 24 \( \mu \)g of DTAT13 given as 2 \( \mu \)g/injection every other day for 3 days without any toxic effects. In contrast, deaths occurred in groups of mice (n = 5/group) given a total of 6 \( \mu \)g of DTIL13 (given as 2 \( \mu \)g/injection every other day for 3 days). The most toxic IT injected intracranially was DTAT. The greatest concentration of DTAT that mice (n = 10/group) tolerated was 0.15 \( \mu \)g given every other day for 3 days.

Together, studies in which IT was injected by both the intratumoral and intracranial routes indicated that DTAT13 was at least 160-fold less toxic than DTAT and at least 8-fold less toxic than DTIL13.
To study the toxic effects of the tumor treatment dose of DTAT13, mice with flank U373 tumors were given the same 10 mg/dose subcutaneous treatment schedule as described previously. Frozen tissue sections were stained with H&E and examined microscopically. Kidney tissue appeared unaffected by DTAT13 treatment with the exception of some minor neutrophil infiltration. Figure 10A shows that glomeruli appeared healthy. These studies were particularly important because similar doses of IT in other studies (Vallera et al., 1997) have induced glomerular destruction, rupture of renal tubules and proximal tubular vacuolization. Liver effects were studied in the same group of mice (Figure 10B). Examination of tissues revealed a few areas of infiltration. Otherwise, tissues appeared relatively unaffected by treatment. Brain, spleen and skin appeared unaffected by treatment. Also, histology did not reveal any liver or kidney damage in mice given IT intracranially.

**Discussion**

The major contribution of this work is the design and synthesis of a new recombinant anti-glioblastoma agent, DTAT13, that recognizes two unrelated determinants on the surface of glioblastoma cells. Our group previously described DTAT and DTIL13 fusion proteins and independently reported their ability to inhibit the growth of glioblastoma tumor cells, particularly as solid tumors in the flanks of nude mice (Li et al., 2002; Vallera et al., 2002). Although both agents were highly effective, they differed markedly with regards to the cell surface marker they targeted. For example, patient glioblastoma cells that express high levels of IL13R would be readily killed by DTIL13. Those cells that do not express high levels of IL13R would not be killed as well. Also, it could be anticipated that some lines that do not express high levels of IL13R would not be killed as well. Also, it could be anticipated that some lines that do not express high levels of IL13R would not be killed as well. Also, it could be anticipated that some lines that do not express high levels of IL13R would not be killed as well. Also, it could be anticipated that some lines that do not express high levels of IL13R would not be killed as well. Also, it could be anticipated that some lines that do not express high levels of IL13R would not be killed as well. Also, it could be anticipated that some lines that do not express high levels of IL13R would not be killed as well. Also, it could be anticipated that some lines that do not express high levels of IL13R would not be killed as well. Also, it could be anticipated that some lines that do not express high levels of IL13R would not be killed as well. Also, it could be anticipated that some lines that do not express high levels of IL13R would not be killed as well. Also, it could be anticipated that some lines that do not express high levels of IL13R would not be killed as well. Also, it could be anticipated that some lines that do not express high levels of IL13R would not be killed...
faster when multiple receptors were targeted, which may be an added advantage. This may or may not influence the kinetics of inhibition and studies are under way to measure the kill rate of DTAT13 in comparison with DTAT and DTIL13. Why would we synthesize DTAT13 when it is possible to treat with a mixture of DTAT and DTIL13? Our studies indicate that the toxicity is due to the DT moiety. Therefore, we would be administering less toxin if we made a molecule with two ligands and one toxin on the same protein. Studies are under way comparing DTAT13 administration with the administration of mixtures of the individual DTIL3 and DTAT IT. Also, we have identified glioblastoma lines that are killed by both DTAT and DTIL13. This means that some glioblastomas have both sets of markers and, thus, more receptor molecules are available to bind DTAT13.

DTAT and DTIL13 are highly effective and do not appear to show major differences in their ability to kill target cells in vitro and their ability to inhibit the growth of established tumors in vivo. However, one important advantage of the DTAT13 molecule is that the addition of the ATF of urokinase bestows the ability on the molecule to target the tumor neovasculature rather than only overexpressed receptor on the surface of tumor cells. Targeting the neovasculature is a valid therapeutic approach, as evidenced by the studies of several investigators exploring the potential of using IT against endothelial cells (Huang et al., 1997; Olson et al., 1997; Arap et al., 1998). For example, Olson et al. (1997) have assembled a vascular endothelial growth factor (VEGF)–diphtheria IT and shown that it has great potential. VEGF plays an important role in tumor neovascularization and the development of tumor stroma and VEGFR is overexpressed in the endothelial cells of the tumor vasculature. VEGF–diphtheria IT had a powerful anti-tumor effect that is a consequence of selective vascular-mediated injury at the tumor site. In the brain this may be a putative advantage since vascular targeting may come at a cost. The brain has a low tolerance threshold for bleeding and it is possible that vessel damage may cause intracranial bleeding and the risk of clot formation. At the same time there is reason to believe that endothelial cells in the tumor vasculature may be targeted and normal endothelial cells spared. Immunohistochemical studies revealed that uPAR is mostly expressed in tissues undergoing extensive remodeling, e.g. in trophoblast cells in the placenta, in keratinocytes at the edge of incisional wounds and in cells of primary tumor and metastasis (Solberg et al., 2001). Also, uPAR expression may be differentially high in the proliferating endothelium.

DTAT13 was less toxic than DTAT or DTIL13 when injected subcutaneously or intracranially, at least 160-fold less toxic than DTAT and 8-fold less toxic than DTIL13. The enhanced size of DTAT13 may pose an additional advantage. Investigators have reported that smaller recombinant immunotoxins may be filtered into the kidney or present an increased risk of hepatic toxicity. Investigators have shown that a recombinant IT of 60 kDa made with DT390, much like the IT reported here, was dose limited by its toxic side effects (Vallera et al., 1997). When the same molecule was dimerized by introducing a C-terminal cysteine, the toxicity was significantly reduced and larger amounts of protein were tolerated (Vallera et al., 2000). Larger proteins may present a reduced risk of toxicity because they have more favorable pharmacokinetics.

Interestingly, treatment of a group of mice with DTAT13 delayed the relapse of U87 tumors compared with mice treated with DTAT (Figure 8A). Although more mice need to be tested...
to confirm this observation, these studies suggest that in certain circumstances DTAT13 may even perform better than DTAT. Numerous explanations for the observation are possible, but the ability of DTAT13 to recognize the vasculature in addition to markers over-expressed on the tumor could explain it. Further studies are ongoing.

Having demonstrated the efficacy of DTAT13 in the flank tumor model, future experiments must focus on determining whether there are any real advantages of bi-ligand targeting in a model that more closely approximates the clinical use of this agent, i.e. intracranial delivery. Intracranial models have been reported previously (Go et al., 1997; Engebraaten et al., 2002) and we have recently established one. Now it will be important to determine if this different route of inoculation will have an impact on the efficacy and toxicity of DTAT13 in comparison with DTAT and DIL13.

Acknowledgements

We thank Dr Angela Panoskaltsis-Mortari for histology assistance and helpful comments on the manuscript and Dr Robert Kreitman for discussions on urokinase receptor targeting.

References


Received October 3, 2003; revised January 29, 2004; accepted February 2, 2004

Edited by Paul Carter