Targeting glioblastoma multiforme with an IL-13/diphtheria toxin fusion protein in vitro and in vivo in nude mice

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Fusion proteins composed of tumor binding agents and potent catalytic toxins show promise for intracranial therapy of brain cancer and an advantage over systemic therapy. Glioblastoma multiforme (GBM) is the most common form of brain cancer and overexpresses IL-13R. Thus, we developed an interleukin-13 receptor targeting fusion protein, DT390IL13, composed of human interleukin-13 and the first 389 amino acids of diphtheria toxin. To measure its ability to inhibit GBM, DT390IL13 was tested in vitro and found to inhibit selectively the U373 MG GBM cell line with an IC50 around 12 pmol/l. Cytotoxicity was neutralized by anti-human-interleukin-13 antibody, but not by control antibodies. In vivo, small U373 MG glioblastoma xenografts in nude mice completely regressed in most animals after five intratumoral injections of 1µg of DT390IL13 q.o.d., but not by the control fusion protein DT390IL-2. DT390IL13 was also tested against primary explant GBM cells of a patient’s excised tumor and the IC50 was similar to that measured for U373 MG. Further studies showed a therapeutic window for DT390IL13 of 1–30 µg/injection and histology studies and enzyme measurements showed that the maximum tolerated dose of DT390IL13 had little effect on kidney, liver, spleen, lung and heart in non-tumor-bearing immunocompetent mice. Together, these data suggest that DT390IL13 may provide an important, alternative therapy for brain cancer.

Keywords: diphtheria toxin/glioblastoma multiforme/immunotoxin/interleukin-13/toxin fusion protein

Introduction

Glioblastoma multiforme (GBM) is an incurable, heterogeneous, high-grade astrocytic glioma, believed to originate from glial non-neuronal cells (Kleihues et al., 1995). The 2-year survival rate for patients is <20% (Davis et al., 1998). Effective treatment has been hindered by the lack of tumor-specific markers in the majority of patients.

Interleukin-13 (IL-13) (McKenzie et al., 1993; Minty et al., 1993), secreted by activated type-2 T cells and mast cells (Brown et al., 1989), is a pleiotropic lymphokine regulating inflammatory and immune responses. It modulates human monocyte and B cell function but not that of T cells (Zurawski and de Vries, 1994). Besides its presence on normal B cells and monocytes, IL-13 receptor (IL-13R) has been found to be overexpressed on cultured human GBM cell lines and surgical GBM specimens but is not detectable on normal brain (Debinski et al., 1995a, 1999; Debinski and Gibo, 2000). IL-13 shares many functions with IL-4, not only because their function-critical regions are homologous (Minty et al., 1993), but also because of the close relationship between their receptors. Studies show that IL-13R on normal tissues consists of IL-13R alpha1 chain which requires heterodimerization with the IL-4R alpha chain to bind IL-13 with high affinity. On the other hand, IL-13R on GBM as well as on renal and colon carcinoma cells possesses IL-13R alpha2 chain which is non-signaling, IL-4 independent, capable of binding IL-13 with high affinity (Debinski et al., 1999, 2000; Debinski and Gibo, 2000; Joshi et al., 2000; Liu et al., 2000). Since IL-13R alpha2 chain may function as a tumor-specific, high-affinity target, targeting IL-13R with a highly toxic agent is a beneficial strategy.

A number of recombinant toxin fusion proteins (FP) consisting of catalytic toxins spliced to binding proteins have been reported (Pastan et al., 1992). However, one of the major clinical obstacles to such agents given systemically is their toxicity to non-target tissues (Foss et al., 1994). In contrast, targeted therapy of brain tumors via intracranial injection has produced responses that are both striking and encouraging (Kunwar et al., 1993; Philips et al., 1994; Laske et al., 1997; Rand et al., 2000). For example, transferrin (Tf) receptor has been targeted using Tf-CRM107, a conjugate of human transferrin and a genetic mutant of diphtheria toxin (DT) that lacks native toxin binding capability (Laske et al., 1994). In a phase I clinical trial, 60% of GBM patients showed at least a 50% reduction in tumor volume without symptomatic systemic toxicity (Laske et al., 1997). This is mostly because direct intratumoral administration circumvents the major problems of systemic administration. These include inefficient distribution to tumor site related to the distance traveled and unfavorable tumor vasculature dynamics (high interstitial pressures) (Jain, 1989). Consequently, intratumoral therapy guarantees more consistent delivery of FP to the targeted site with limited systemic exposure, which results in less toxicity.

We chose the DT390 truncation of diphtheria toxin to construct our immunotoxin because Williams and co-workers described a series of internal in-frame deletion mutations that established 389 as the optimum site for genetic fusion of DT and targeting ligands (Williams et al., 1990). In our own experience, fusion toxins made with this mutation retain full enzymatic and translocation enhancing activity, but exclude the native DT binding domain (Chan et al., 1996; Vallera et al., 1996, 2000a). IL-13R was chosen in our study because besides GBM, IL-13R is overexpressed on many human malignancies including renal cell carcinoma (Obiri et al., 1995), ovarian carcinoma (Murata et al., 1997), colon adenocarcinoma (Debinski et al., 1995b), epidermoid carcinoma (Debinski et al., 1995b), AIDS-associated Kaposi’s sarcoma (Obiri et al.,...
1997), prostate carcinoma (Maini et al., 1997) and pancreatic cancer (Kornmann et al., 1999). Furthermore, an IL-13-based Pseudomonas exotoxin A (PE) FP showed promising antitumor activity in vitro and in vivo against various human malignancies besides GBM (Debinski et al., 1995a,b, 1998; Puri et al., 1996; Husain et al., 1997, 2001; Maini et al., 1997; Kornmann et al., 1999; Husain and Puri, 2000) but not toxicity to normal endothelial, lymphoid or bone marrow precursor cells (Puri et al., 1996; Husain et al., 1997). DT was chosen as a toxin for our studies because one molecule of DT enzymatic domain in the cytoplasm is sufficient to kill a cell (Yamaizumi et al., 1978). Also, since all toxin FP are immunogenic and in cases where treatment ultimately results in an anti-toxin response, DT390IL13 would be available as an alternative therapy. Since IL-13 cross-reacts among species (Zurawski et al., 1995), we evaluated both efficacy and toxicity of human IL-13 FP in mouse models. These studies showed that it is highly efficacious and has an acceptable therapeutic window.

Materials and methods

Production of recombinant DT390IL13

The cytokine fusion toxin gene was assembled using DNA fragments encoding human IL-13 spliced to DT 390 by the method described previously (Vallera et al., 1996). The hybrid gene was ligated into expression vector pET21d (Invitrogen, Carlsbad, CA). Successful cloning of the designed gene was confirmed by restriction endonuclease digestion and sequencing analysis at the University of Minnesota Microchemical Facility (Minneapolis, MN). Plasmid pDThIL13.pET21d was then transformed into the Escherichia coli strain BL21(DE3) (Novagen, Madison, WI). Expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (GIBCO, Gaithersburg, MD) and protein from inclusion bodies was solubilized, refolded and purified as described previously (Vallera et al., 1996). The purified fusion protein DT390IL13, M, 55 539, was then analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) on a 10% non-reducing gel (Bio-Rad, Richmond, CA) and a Mini-protein II gel apparatus (Bio-Rad). Control DT based FP used in these studies have been reported previously (Williams et al., 1987; Vallera et al., 1996, 2000a).

Cell lines and antibodies

The cell lines U87 MG, U373 MG and T98 G, established from human GBM, were kindly provided by Dr Walter Low, Department of Neurosurgery, University of Minnesota and are available from ATCC (Rockville, MD). HUT-102, derived from human T cell lymphoma, and C1498, originating from murine leukemia, were also from ATCC. They were all maintained in RPMI 1640 medium (Biowhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Biowhittaker), 2 mM L-glutamine (GIBCO), 0.1 mM non-essential amino acids (GIBCO), 1.0 mM sodium pyruvate (GIBCO), 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO). All the cells were maintained at 37°C in a humidified incubator of 5% CO2–95% air and passaged twice per week.

Polyclonal anti-human IL-13 antibody (Cat. No., AF-213-NA; Lot No., MZ04; Ig class, goat IgG) and monoclonal anti-human IL-4R antibody (Cat. No., MAB230; Clone, 25463.1; Lot No., MB029101; Ig class, mouse IgG2a), both obtained from R&D Systems (Minneapolis, MN), were used for the blocking studies. Anti-murine IL-4 antibody from clone 11B11 rat IgG1 (Ohara and Paul, 1988) was used as a control.

Primary explants of GBM

A fresh diagnosed human GBM tumor surgical specimen was provided by the Department of Neurosurgery, Fairview University Hospital (Minneapolis, MN). The sample was minced into 1–2 mm pieces and resuspended in 0.05% trypsin–0.53 mM EDTA (GIBCO) at 37°C for 30 min (Lolait et al., 1983). The tumor tissue suspension was washed in RPMI-10, the same supplemented RPMI medium used for cell culture in this study and pelleted at 1200 r.p.m. for 5 min. The pellet was then resuspended in fresh RPMI-10, plated and incubated at 5% CO2 at 37°C. On the second day, culture medium and non-adherent materials were discarded by gently washing the flask with fresh medium once. The culture was assayed after one passage.

In vitro cytotoxicity assay

Inhibition of cellular DNA synthesis was measured to indicate the cytotoxicity of DT390IL13. Cells were plated at 104 cells/well in 100 µl of RPMI-10 in a 96-well flat-bottomed tissue culture plate and incubated overnight at 37°C. Volumes of 100 µl of various concentrations of DT390IL13 or control FP, diluted in RPMI-10, were added to the cells and incubated for 24, 48 or 72 h. Levels of 1 µCi of [methyl-3H]thymidine (Amersham Pharmacia Biotech UK, Amersham, Bucks, UK) were added to the wells at the beginning of the last 8 h of incubation. At the end of incubation, the cells were washed and harvested on glass-fiber filters and incorporation of radioactivity was quantitated. Data were obtained from the average of triplicates and expressed as a percentage of incorporated [methyl-3H]thymidine counts for cells incubated with only culture media. The assays were performed at least twice. The concentration of DT390IL13 at which 50% inhibition of DNA synthesis (IC50) occurred was calculated. For neutralization experiments, antibodies were preincubated with cells or FP for 30 min and then the assay was performed as described above.

Mice

Female homozygous athymic nu/nu nude mice and C57BL/6 mice, 6 weeks old, were purchased from NIH (Bethesda, MD) and maintained in microisolator cages in a specific pathogen-free facility at the Department of Research Animal Resources, University of Minnesota. Sentinel mice are maintained in the facility and routinely monitored for MHV, Sendai and other infectious viruses.

Nude mouse flank tumor model

On days -4 and -2, 25 µl of anti asialo GM1 (Rabbit) (Wako Chemicals USA, Richmond, VA, Cat. No. 986–10001) diluted in 175 µl of PBS were injected intraperitoneally (i.p.) into each nude mouse. It inhibits mouse NK cells, monocytes and fetal thymocytes, thus potentiating tumor growth by inhibiting rejection of tumor. On day 0, 6×106 U373 MG cells resuspended in 0.1 ml of RPMI-10 were injected subcutaneously (s.c.) into the right flank of each nude mouse. Tumor size was measured by caliper every 2–3 days. The approximate tumor volume was calculated as a product of the three measurements, two perpendicular diameters and the height. Mice were observed for visible toxic signs every day and animal body weight was recorded every 2 days. Volumes of 50 µl of DT390IL13 diluted in sterile PBS were injected intratumorally (i.t.) from three different directions through a 0.5 ml insulin syringe. Treatment was repeated every other
Fig. 1. Construct encoding the DT390IL13 gene fragment used in these studies. The 1.5 kb hybrid gene containing an ATG initiation codon, the first 389 amino acids of DT, a seven amino acid linker EASGGPE and the 109 amino acid mature peptide of human IL-13 was cloned into the pET21d expression vector at Ncol/Xhol sites resulting in the plasmid pDT390hIL13.pET21d.

Fig. 2. SDS–PAGE of the purified DT390IL13 protein stained with Coomassie Brilliant Blue. Lane 1, molecular weight standard; lane 2, DT390IL13 following column purification; lane 3, DT390IL2 following column purification. The molecular weight standards were 97.4, 66, 45 and 31 kDa.

day (q.o.d.) for a total of five doses. Animals were randomly assigned to treatment groups. Groups received either DT390IL13, control DT390IL2 (human) or PBS.

Blood urea nitrogen (BUN) and alanine transferase (ALT) assays

DT390-anti-CD3sFv is known to cause severe renal toxicity in mice (Vallera et al., 1997). Therefore, DT390-anti-CD3sFv, DT390IL13 or sterile PBS was injected s.c. into the right flank of 10-week-old C57BL/6 mice in a 100 µl volume for five q.o.d. doses. Mice were killed 24 h after the last dose and individual serum samples were obtained from blood via heart puncture. Analysis was performed in coded fashion on the undiluted serum samples. As described previously (Vallera et al., 1997), both assays were performed on a Kodak ETA-CHEM 950 by the Clinical Chemistry Laboratory, Fairview University Medical Center, University Campus (Minneapolis, MN).

Histology study

Tissue specimens of heart, lung, spleen, liver and kidney were taken from the same mice whose sera were used for blood BUN and ALT assays. Histology studies were performed as described previously (Vallera et al., 1997). All samples were embedded in OCT compound (Miles, Elkhark, IN), 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).

Statistical analysis

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

Results

Construction and purification of DT390IL13

The assembled plasmid pDThIL13.pET21d under the control of the IPTG inducible T7 promoter is illustrated in Figure 1. DNA sequencing analysis (University of Minnesota Microchemical Facility) verified that the hybrid gene had been cloned in frame. Expression of DT390IL13 in E.coli was induced with IPTG at 37°C. SDS–PAGE analysis of the purified, refolded, Coomassie Brilliant Blue-stained fusion protein showed a protein at ~58 kDa, the expected size for DT390hIL13 (Figure 2). Both DT390IL13 and DT390IL2 were >95% pure.

DT390IL13 is selectively cytotoxic to human GBM cell lines

To determine the potency of DT390IL13, various cell lines were cultured in the presence of increasing concentrations of DT390IL13 for 24, 48 and 72 h. Cells were pulsed with [methyl-3H]thymidine and the radioactivity was counted. Human GBM cell lines U373 MG, U87 MG and T98 G and control cell lines C1498 and HUT-102 which do not express IL-13R were examined (Figure 3A). Previous studies showed that U373 MG had ~16 400 IL-13R/cell whereas T98 G had only 549 (Debinski et al., 1995a). The density of IL-13R on U87 MG is intermediate between those two cell lines (Debinski et al., 1996). Figure 3A shows the dose-dependent killing of U373 MG cells by DT390IL13 with IC50 around 12 pM and 1 nM, respectively. These IC50 were negatively correlated with the density of IL-13R expression. Non-IL-13R-expressing cell lines C1498 and HUT-102 and low IL-13R expressing T98 G were not killed by DT390IL13 even at higher concentrations. In Figure 3B, control FP DT390IL2 (Kirkman et al., 1989) and DT390-mIL4 (Vallera et al., 2000b) showed no cytotoxicity against U373 MG cells even at high concentrations though they were highly toxic against HUT-102 and C1498, respectively. Figure 3C shows neutralization of the cytotoxicity of DT390IL13 against U373 MG cells by treating with polyclonal anti-human IL-13 antibody at 1.0 µg/ml but not by treating with monoclonal anti-human IL-4R antibody or 11b11 (antimurine IL-4 antibody) at the same concentration. Studies showed that IL-13 also binds IL-4R on GBM cells (Debinski et al., 1996). At a high concentration, anti-human IL-4R did not alter the cytotoxicity of DT390IL13 in our study, thereby indicating that the cytotoxicity of DT390IL13 was mediated through IL-13R but not IL-4R. Together, these data demonstrate that DT390IL13 is highly selective in inhibiting IL-13R over-expressing cells in vitro.

DT390IL13 kinetics

We performed kinetic studies to determine whether cytotoxicity of DT390IL13 can be enhanced by prolonged exposure for 24, 48 and 72 h. Figure 4A shows that the cytotoxicity of DT390IL13 against U373 MG cells was maximum at 48 h.

IL13/diphtheria toxin fusion protein
incubation and longer incubation periods did not enhance the cytotoxicity. Prolonged exposure to DT$_{390}$IL13 did not alter the susceptibility of low IL-13R expressing control T98 G cells shown in Figure 4B. Thus, the maximum level of killing was achieved at 48 h.

In vivo anti-U373 MG tumor activity of DT$_{390}$IL13 in nude mouse model
To determine the efficacy of DT$_{390}$IL13 in vivo, a nude mouse xenograft model was employed. U373 MG cells were inoculated s.c. into the right flank of nude mice. Twelve days later, groups of mice were treated i.t. with 1 µg of DT$_{390}$IL13, 10 µg of DT$_{390}$IL13 and 10 µg of DT$_{390}$IL2 or PBS. Figure 5A shows the individual tumor growth curves for five mice treated with 10 µg/dose of DT$_{390}$IL13. All tumors regressed following a regimen of five q.o.d. doses. In Figure 5B, the curves in Figure 5A are pooled and compared against a control group of mice given PBS. The anti-tumor effect of DT$_{390}$IL13 was highly significant ($p < 0.005$). In Figure 5C, two of three tumors given as little as 1 µg of DT$_{390}$IL13 still regressed. In contrast, tumors in a control group given DT$_{390}$IL2 continuously grew over the study period. Statistically significant differences in tumor size were detectable when the 10 µg/dose DT$_{390}$IL13 group was compared with the control PBS group ($p < 0.005$) and the 1 µg/dose DT$_{390}$IL13 group was compared with the DT$_{390}$IL2 group ($p < 0.02$). The tumor size differences between the 1 and 10 µg/dose DT$_{390}$IL13 groups were not significant over the study period. The body weights of mice treated with DT$_{390}$IL13 did not change significantly during the course of treatment. These data show that as little as 1 µg/dose of DT$_{390}$IL13 was able to cause an anti-tumor response.

To reproduce our findings, a group of five mice were administered 10 µg/dose DT$_{390}$IL13 exactly as in Figure 5. Individual tumor growth curves are shown for each experimental animal in Figure 6. Again, all mice responded and all of the tumors initially regressed. Two of the five tumors which were the smallest in size were cured. The other three tumors relapsed. One of these mice bearing the relapsed tumor died early of unknown causes. The other two tumors were retreated on day 77 (tumor size = 0.2–0.4 cm$^3$) with a 5-day course of 10 µg/dose DT$_{390}$IL13 given q.o.d. Tumor growth stabilized during treatment, but then resumed. Data suggest that the tumors become resistant to multiple courses of DT$_{390}$IL13. This resistance is likely not attributable to an anti-DT$_{390}$IL13 immune response since the study was performed in nude mice.

Determination of the maximum tolerated dose (MTD) of DT$_{390}$IL13
Non-tumor-bearing C57BL/6 mice ($n = 6$ or 7 per group) received s.c. injections of 30 or 60 µg/dose DT$_{390}$IL13 q.o.d. One of six mice receiving 60 µg/dose died after the second...
dose and had lost 15% body weight, whereas the other mice survived after all doses and did not show any signs of toxicity even 18 days after the last dose. In the 30 µg/dose group, all seven mice survived and did not show any signs of toxicity even 10 days after the last dose, indicating the MTD. Therefore, we concluded that the therapeutic window is defined as the safe dosages between the efficacious dose and the MTD or, in the case of DT₃₉₀IL₁₃, 1–30 µg/dose.

Systemic toxicity of DT₃₉₀IL₁₃

To evaluate the systemic toxicity of DT₃₉₀IL₁₃, non-tumor-bearing C57BL/6 mice (n = 5/group) were given five q.o.d. injections of DT₃₉₀IL₁₃ at 30 µg/dose, 20 µg/dose DT-anti-CD₃sFv or PBS with the same schedule as the nude mice study. Human IL-13 is species cross-reactive so DT₃₉₀IL₁₃ does react with mouse IL-13R. Frozen tissue specimens of kidney, liver, spleen, lung and heart were sectioned, stained and examined. For the DT₃₉₀IL₁₃ group, none of the examined tissues showed apparent pathological changes except some neutrophil and mononuclear cell infiltration and aggregation in the kidney (Figure 7A) and liver (Figure 7B). Endothelial cells in these organs showed no pathological changes at the light microscope level. Furthermore, Figure 8B shows that the serum levels of BUN were not significantly elevated in the DT₃₉₀IL₁₃ group compared with controls and confirmed that no significant kidney damage occurred. DT₃₉₀-anti-CD₃sFv causes severe renal dysfunction (Vallera et al., 1997) so it was used as a control in this study. The DT₃₉₀-anti-CD₃sFv group showed dramatic pathological damage in the kidney. The significant elevation of serum levels of BUN (p = 0.02) shown in Figure 8B indicates damage to the kidney. Both of the FP caused significantly elevated serum levels of ALT, shown in Figure 8A, indicating that the liver function may be slightly affected by the administration of these two FP. Together, these data suggest that DT₃₉₀IL₁₃ at the MTD did not severely affect organ functions.

DT₃₉₀IL₁₃ is also cytotoxic to GBM explant cells

DT₃₉₀IL₁₃ kills receptor-overexpressing established GBM cell lines, but these lines may have different receptor expression characteristics to primary explanted cells. Thus, activity of DT₃₉₀IL₁₃ was measured on primary explanted GBM cells. Table I shows that the human explanted GBM cells are sensitive to the action of DT₃₉₀IL₁₃. However, the IC₅₀s are variable consistent with the argument that clinical GBMs vary with regard to their expression of IL13 receptor.

Discussion

The major contribution of this study is the description of a new fusion protein, DT₃₉₀IL₁₃, which shows promise for clinical use against GBM. We have shown for the first time that truncated diphtheria toxin can be used as a toxin moiety to synthesize a highly potent and specific human IL-13 based anti-glioblastoma agent. The effect of DT₃₉₀IL₁₃ was determined in vitro on GBM cell lines and also on primary explant cells of surgical human GBM specimens from our institution and results indicate that the efficacy of DT₃₉₀IL₁₃ is not limited to cultured cell lines. Our results show that DT₃₉₀IL₁₃ is highly specific, both in vitro and in vivo, with a workable therapeutic window and acceptable toxicity. Specificity of DT₃₉₀IL₁₃ was shown by blocking the effects of DT₃₉₀IL₁₃ with anti-IL-13 antibody, but not with irrelevant control antibody. Also, control cytokine FP that did not recognize the IL13R was unable to inhibit U373 MG tumor growth in nude mice. Also, these data indicate that patient explant cells are not all equally sensitive to DT₃₉₀IL₁₃ so that it may be necessary to develop other IT also.

Another important aspect of these studies is the measurement of a favorable, albeit narrow, therapeutic window of DT₃₉₀IL₁₃ of 1–30 µg. IL-13R is not unique to neoplastic diseases: it is present in normal B cells, monocytes, endothelials and hematopoietic progenitor cells at low levels. Our study demonstrated that at the MTD, no apparent organ toxicities were detected in heart, lung, spleen, liver and kidney of mice. Other studies showed that normal resting or activated human T, B and monocytic cells, lymphoid, bone marrow precursor cells

Fig. 4. Kinetics of DT₃₉₀IL₁₃ killing of U373 MG and T98 G. Cells were cultured with FP for 24, 48 or 72 h and then assayed for tritiated thymidine incorporation. (A) data measuring the activity of DT₃₉₀IL₁₃ against U373 MG; (B) data measuring the activity of DT₃₉₀IL₁₃ against T98 G. Data are presented as percentage of control response (mean of triplicates ± one standard deviation). Control values measuring the proliferation of untreated U373 MG at 24, 48 and 72 h are 12 233 ± 1508, 24 896 ± 3083, 37 384 ± 2005 c.p.m., respectively; for T98 G they are 19 906 ± 1725, 46 168 ± 6248, 90 384 ± 1771 c.p.m., respectively.
Table I. Activity of DT\textsubscript{390}IL13 against primary GBM explant cells

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Passage number</th>
<th>IC\textsubscript{50} (nM DT\textsubscript{390}IL13)</th>
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<tr>
<td>1</td>
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<td>2</td>
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Primary explant cells originating from three patients diagnosed with GBM were cultured immediately following surgery. The explant cells, within the second passage, were cultured with DT\textsubscript{390}IL13 for 48 h and then proliferation was measured using a tritiated thymidine incorporation assay. Data are represented as the IC\textsubscript{50} or concentration of DT\textsubscript{390}IL13 that inhibits 50% of the control response. Control values ranged from 1207 ± 204 to 3633 ± 185 c.p.m.

and normal human umbilical vein-derived endothelial cells were not susceptible to IL13-PE38QQR (Puri et al., 1996; Husain et al., 1997). Although other explanations are possible, this can be explained by different levels of expression of IL-13R on normal tissues.

Evidence suggests that IL-13R and IL-4R share at least one common subunit, IL-4R 140 kDa alpha chain with exceptions found only in GBM cells, normal testes, some renal and colon carcinoma cells (Debinski et al., 1999, 2000; Debinski and Gibo, 2000; Joshi et al., 2000; Liu et al., 2000). In our studies, DT\textsubscript{390}IL13 activity was IL4-independent since (1) anti-hIL-4R antibody failed to block DT\textsubscript{390}IL13 and (2) DT\textsubscript{390}IL13 did not inhibit the proliferation of T98 G and HUT-102, both of which are known to overexpress human IL-4R (Debinski et al., 1993).

Others have reported that a PE-based IL-13 FP, namely IL13-PE38QQR, can be used to kill glioblastoma and other IL-13R-overexpressing human malignancies both in vitro and in vivo (Debinski et al., 1995a,b, 1998; Puri et al., 1996; Husain et al., 1997, 2001; Maini et al., 1997; Kornmann et al., 1999; Husain and Puri, 2000). PE is immunogenic so it may be possible to use DT\textsubscript{390}IL13 as an immunotoxin to circumvent an immune response. Eventually, DT would also elicit an immune response, so the use of non-immunogenic toxic moieties such as ribonucleases (Suzuki et al., 1999) or immunotoxins combined with chemotherapy may be important in future drug development.

Owing to the blood–brain barrier, intratumoral delivery of FP for central nervous system (CNS) tumors is advantageous. Because GBM is not generally a systemic disease, intratumoral delivery of FP results in the FP compartmentalization inside the tumor. Experiments with radiolabeled DT\textsubscript{390}IL13 showed that DT\textsubscript{390}IL13 was remarkably localized to the tumor via the intratumoral route (D.A.Vallera, unpublished data). Local delivery may allow higher dosages of FP in order to achieve complete remission since it avoids the toxic effects that may be associated with systemic exposure. The immunoprotective

Fig. 5. DT\textsubscript{390}IL13 administered intratumorally causes the regression of small established U373 MG tumors in nude mice (n = 4–5/group). Six million U373 MG cells were injected subcutaneously into nude mice. On day 12 after tumor inoculation, mice were given five q.o.d. intratumoral injections of FP. The treatment duration is indicated by the heavy solid line on the abscissa. Data are presented as tumor volume (cm\textsuperscript{3}) plotted versus time. (A) Tumor growth curves of individual mice given 10 µg/dose DT\textsubscript{390}IL13 group; (B) tumor growth curve averages of groups of mice given 10 µg/dose DT\textsubscript{390}IL13 or PBS as a control; (C) tumor growth curve averages of groups of mice given 1 µg/dose DT\textsubscript{390}IL13 group or 10 µg/dose DT\textsubscript{390}IL2 as a control. Error bars represent the mean ± one standard error unit. Time periods with significant p values are indicated.
environment of the CNS may also allow for repeated administration of FP without the development of neutralizing antibodies, those antibodies that block binding of the FP to the targeted receptor. So with regard to the blood–brain barrier, potential organ toxicities and antigenicity, DT$_{390}$IL13 may be especially efficacious in the treatment of CNS tumors.

IL-13 is species cross-reactive so that the human IL-13 used for the assembly of DT$_{390}$IL13 was also reactive with mouse IL-13 receptor. Furthermore, the distributions of murine and human IL-13R alpha1 chain are similar among normal tissues (Aman et al., 1996; Hilton et al., 1996). To evaluate better the toxicity of DT$_{390}$IL13, we used non-tumor-bearing C57BL/6 mice instead of nude mice. Normal mice would have the B cells lacking in nude mice and these B cells also express IL-13R and thus represent a potential reservoir for FP. Non-tumor-bearing C57BL/6 mice tolerated dosages of 30 µg/dose, 30-fold higher than the 1 µg/dose that induced anti-tumor responses. It is likely that higher doses may be tolerated in animals with larger tumors because larger tumors provide a larger antigen sink and can soak up more DT$_{390}$IL13. However, larger tumors reduce the possibility of thorough diffusion of FP throughout the entire tumor bed, which could lead to partial or no tumor response at all. Improved diffusion of FP can be achieved by higher dose, more frequent administration and more injection directions. This issue is currently under study. Also, continuous i.t. delivery of FP through implanted infusion pump may be helpful for larger tumors to achieve complete tumor response. In our preliminary studies, nude mice bearing large U373 MG tumors tolerated as much as five q.o.d. 100 µg/dose of DT$_{390}$IL13 and demonstrated tumor response by a 50% reduction in volume of one large tumor (D.A. Vallera, unpublished data). Thus, the therapeutic window of DT$_{390}$IL13 is at least 30-fold for small tumors and possibly higher for large tumors.

In one experiment, despite initial tumor regression, relapse occurred and tumor appeared more refractory to subsequent DT$_{390}$IL13 treatment. This was not due to the immune response since the study was performed in nude mice. We favor the explanation of down-regulated IL-13R on tumor cells and studies are currently examining this issue. DT$_{390}$IL13 appeared to be well tolerated compared with DT$_{390}$-anti-CD3sFV, which caused renal dysfunction at much lower doses than DT$_{390}$IL13 in these studies. Rapid clearance and filtration of this 60 kDa FP into the kidney were thought to cause the renal toxicity and dimerized DT-anti-CD3sFV was much better tolerated than monomer, possibly because the larger size reduced the kidney clearance (Vallera et al., 2000b). However, similarly sized DT$_{390}$IL13 did not damage renal histopathology even at 30 times the efficacy dose. Furthermore, the 58 kDa DT$_{390}$-mIL3 did not cause significant damage to kidney or liver even at its MTD (Vallera et al., 1999). These data suggest that the size of FP alone does not predict whether renal toxicity will occur.

![Fig. 6](image1.jpg)

**Fig. 6.** DT$_{390}$IL13 administered intratumorally is not effective against tumors in animals given multiple courses. In an additional animal experiment, groups of mice were given five q.o.d. injections of 10 µg/dose DT$_{390}$IL13 as in Figure 5. Treatment was begun on day 23. Individual tumor growth curves are shown for each experimental animal. Two tumors regressed and one of these mice bearing the relapsed tumor died early of unknown causes. The other two tumors were retreated on day 77 with a course of five q.o.d. 10 µg/dose DT$_{390}$IL13.

![A](image2.jpg) ![B](image3.jpg)

**Fig. 7.** Histology studies. Non-tumor-bearing C57BL/6 mice were randomly grouped (n = 5/group) and injected subcutaneously with DT$_{390}$IL13, a control FP DT$_{390}$-antiCD3sFV known to cause organ toxicity or PBS based on the same schedule in nude mice experiment. Twenty-four hours after the fifth dose, the mice were killed and hearts, lungs, spleens, livers and kidneys were removed, sectioned and stained with H&E. Three mice per group were examined with identical results. Photographs of (A) kidney and (B) liver from the 30 µg/dose DT$_{390}$IL13 group are shown.
in evaluating DT 390IL13 as a potential anti-brain tumor agent.

Although the flank model has proven informative, our goal is to evaluate DT 390IL13 as a potential anti-brain tumor agent. Thus, a model in which DT 390IL13 can be tested against intracranial tumors could prove more relevant. Studies are currently under way in such a model which will be valuable in establishing dosages and pinpointing toxic side effects, especially those relating to intracranial bleeding.

The GBM field has progressed in the discovery of a large number of different cytokine and growth factor receptors that are overexpressed exclusively on tumor cells. These receptors may serve as selective targets for FP therapy approaches. Various FP have been directed to these receptors, including IL-4R (Debinski et al., 1993; Kreitman et al., 1994; Husain et al., 1998), epidermal growth factor receptor (EGFR) (Kunwar et al., 1993; Phillips et al., 1994) and transferrin receptor (Laske et al., 1994). Since it is more than likely that none of these by themselves may be curative, it may be possible to address a wider range of tumors with several of these agents perhaps used as a cocktail (Vallera et al., 1983). For example, animal studies show that DT 390IL13 at these doses and schedules cannot cure larger tumors and it may be necessary to use other agents. We found that many of the GBM lines that were killed by DT 390IL13 were also killed by an additional anti-GBM agent that we have synthesized, namely DT 390 fused to the N-terminal fragment of urokinase-type plasminogen activator (Vallera et al., 2002). Perhaps targeting different receptors expressed on the same GBM will deliver higher levels of cell kill. Furthermore, it may be possible to combine these toxin FP with chemotherapy or radiation therapy to enhance GBM killing.

In summary, we have demonstrated that DT 390IL13 is highly potent and selective against primary explanted IL-13R 

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In summary, we have demonstrated that DT 390IL13 is highly potent and selective against primary explanted IL-13R 


cells and capable of causing regression of small human GBM tumors grown in the flanks of nude mice without significant toxicity. It will be important to determine whether DT 390IL13 will affect GBM brain tumors in intracranial mice and rat models. The data in this paper indicate that DT 390IL13 may be used as an alternative agent for the therapy of GBM and other IL-13R 

malignancies.

Acknowledgements

We thank Sekou Doumbia for his valuable technical assistance. This work was funded in part by the Minnesota Medical Foundation.

References


IL-13/diphtheria toxin fusion protein


Received August 31, 2001; revised November 29, 2001; accepted February 8, 2002