Interstitial Diphtheria Toxin-Epidermal Growth Factor Fusion Protein Therapy Produces Regressions of Subcutaneous Human Glioblastoma Multiforme Tumors in Athymic Nude Mice

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ABSTRACT

Purpose: The novel fusion protein, DAB389EGF, composed of the catalytic and translocation domains of diphtheria toxin (DAB389) fused with a His-Ala linker to human epidermal growth factor (EGF) was tested for antiglioma efficacy in an in vivo model of human glioma.

Experimental Design: Female athymic nude mice (ages 4–6 weeks) were inoculated s.c. with 10 million U87MG human glioma cells in the right flank. When tumor volumes reached ~100 mm³ (~6–8 days), i.t. injections of saline, DAB389IL2, or DAB389EGF 1, 3, 5 or 10 μg in 50 μL were given every other day for three to six doses. Animals were monitored twice daily and tumor measurements were made by calipers.

Results: The maximal tolerated dose (MTD) of DAB389EGF was 3 μg every other day. Above the MTD, animals experienced loss of activity, reduced oral intake, and dehydration. Blood chemistries confirmed elevated blood urea nitrogen, creatinine, aspartate transaminase, and alanine transaminase. Histopathology revealed renal tubular necrosis. At the MTD, tumor regression was seen in all animals. Relapses occurred in 4 of 16 (25%) of animals after 1 month. These tumors contained EGF receptor, were sensitive in vitro to DAB389EGF, and responded to a second course of i.t. DAB389EGF.

Conclusions: DAB389EGF fusion protein was shown in vivo antiglioma efficacy in a s.c. tumor model and warrants further preclinical testing in an i.c. tumor model for eventual treatment of patients with recurrent or refractory EGF receptor–positive glioblastoma multiforme.

INTRODUCTION

There are 18,000 new cases of primary brain tumors per year in the United States (1). Most patients progress and die from their disease. For the most common variety, glioblastoma multiforme, the median survival is 1 year for newly diagnosed patients and 6 months for patients with recurrent disease (2). Fewer than 20% of glioblastoma multiforme patients are alive at 2 years. These tumors are radiotherapy and chemotherapy resistant (3). Novel agents with different mechanisms of action are needed for brain tumor patients.

One novel class of brain tumor therapeutics is fusion proteins consisting of brain tumor–selective ligands conjugated to peptide toxins. The ligands direct the agent to the glioma cell surface. The peptide toxins then internalize into the cell, translocates to the cytosol, and catalytically inactivates protein synthesis, leading to cell death. A series of brain tumor fusion proteins have been prepared and had antiangioma activity in tissue culture, in animal models, and in patients (4–12). The fusion proteins are delivered to the tumor cells by convection enhanced delivery (CED). Catheters are introduced into the tumor bed or normal brain surrounding the tumor resection cavity (12); continuous fluid pressure over several days is added to create a bulk flow, which supplements diffusion. CED achieves high protein concentrations in a large volume of the tumor and surrounding brain tissue. The protein concentrations are orders of magnitude higher than would be achieved by systemic administration or bolus i.t. injections (13). Clinical remissions lasting years have been observed in many patients (4, 6, 12). Because of toxicities to normal brain tissue and heterogeneity of receptor expression among brain tumors, there is a need for additional brain tumor fusion proteins.

We chose a diphtheria fusion protein composed of the catalytic and translocation domains of diphtheria toxin (DAB389) fused via a His-Ala linker to epidermal growth factor (EGF). DAB389EGF was expressed in Escherichia coli and purified from inclusion bodies as described previously (14). The fusion protein was 48,522-Da, reacted with antibodies to diphtheria toxin and EGF on immunoblots, and was selectively toxic to EGF receptor (EGFR)–overexpressing tumor cell lines xd (15). We showed potent DAB389EGF toxicity to a battery of glioma cell lines (16). Efficacy was proportional to EGFR density. We also examined receptors densities for different fusion proteins among a large set of human brain tumors. EGFR was more frequently expressed and at a higher level than the other examined receptors (17). We wanted to extend this work to an in vivo brain tumor system. In this report, we describe the antiangioma efficacy of DAB389EGF in a human glioma-athymic...
nude mouse xenograft model and better characterize the maximal tolerated dose (MTD) and dose-limiting toxicity of i.t. therapy. Further, we explore models of DAB389EGF-mediated in vivo brain tumor cell death. The results establish that the excellent anti-brain tumor activity of DAB389EGF extended to an interstitial therapy animal model and provide a rationale for CED DAB389EGF therapy of patients with recurrent EGFR positive gliomas.

MATERIALS AND METHODS

Cells. The human glioma cell line U87MG was purchased from the American Type Culture Collection (Rockville, MD) and were grown in Eagle’s medium containing sodium pyruvate, 10% fetal bovine serum, nonessential amino acids, l-glutamine, penicillin/streptomycin, and 1.5 g/L sodium bicarbonate. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO2 in air.

Animals. Female athymic nude mice (ages 4-6 weeks) were obtained from Charles River Laboratories (Wilmington, MA) and BALB/c mice (female, ages 8 weeks) were obtained from Harlan (Indianapolis, IN) and maintained in a ventilated rack system. Irradiated food and autoclaved water were provided ad libitum. Animals were allowed to adjust to their environment for 1 week. The athymic nude mice were then injected s.c. in the right flank with 10 million U87MG cells diluted in 0.1 mL PBS (BioWhittaker, Walkersville, MD). After completion of all injections, cells from residual syringes were >90% viable by trypan blue exclusion. Animals were observed twice daily, and tumor size was measured every other day with calipers. The tumor volume was calculated based on the formula: \( V = \frac{L \times W^2}{2} \), where \( L \) is length and \( W \) is width. Groups of five BALB/c mice received two DAB389EGF or saline s.c. injections of 10 \( \mu \)g every other day and were euthanized and necropsied on day 5.

Blood samples were obtained at the time of euthanasia by cardiac puncture and assayed for serum chemistries.

Drugs. DAB389EGF was synthesized and partially purified as described previously (15). Vials contained 500 \( \mu \)g sterile-filtered, lyophilized DAB389EGF in PBS (pH 7.2) containing 1% mannitol and 50 \( \mu \)mol/L EDTA (Ligand Pharmaceuticals, San Diego, CA). Vials were stored at -80°C. Fusion protein was prepared for experiments by adding 2 mL sterile water and gently stirring. Dissolved protein was stored at 4°C. DAB389IL2 (denileukin diftitox, Ontak) was also obtained from Ligand Pharmaceuticals, diluted in PBS to 0.15 mg/mL, and stored at 4°C.

Necropsies. Mice that presented severely dehydrated, hypothermic, and dyspneic were considered moribund and were euthanized by standard CO2 asphyxiation, consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Blood samples were collected by cardiac puncture and assayed for serum chemistries. All other mice were euthanized before mass size was 1,500 to 3,000 mm³. All mice were killed at day 60 post-tumor cell inoculation. Samples from major organs were removed, fixed in 10% buffered formaldehyde, dehydrated, and embedded in paraffin. Sections were stained with H&E and examined under the microscope.

Efficacy Studies. Groups of 5 to 16 animals received i.t. injections of 1, 3, 5, or 10 \( \mu \)g DAB389EGF, 10 \( \mu \)g DAB389IL2, or saline in 50 \( \mu \)L volume beginning when tumor volume reached 100 mm³ (~6-8 days). Treatments were repeated every other day for 2 weeks. The mice were examined twice daily for overall activity, and bidirectional tumor diameters were measured with calipers every other day for 8 weeks. Moribund mice were killed. Three 3 \( \mu \)g DAB389EGF-treated animals developed recurrent tumors after 1 month and received a second identical course. On day 60, the skin tumor sites of DAB389EGF-treated mice were examined, fixed in 10% buffered formaldehyde, dehydrated, embedded in paraffin, sectioned, and stained with H&E.

Tumor Studies. Tumor samples were collected and divided into three portions. One portion was fixed in 10% buffered formaldehyde, dehydrated, embedded in paraffin, sectioned, and stained with H&E. Another portion was frozen and 6-\( \mu \)m cryostat sections were made and reacted with anti-EGFR or control mouse immunoglobulin and washed, and bound antibody was detected with rhodamine-conjugated goat anti-mouse immunoglobulin as described previously (17). Staining intensities of control and relapsed tumor sections with similar cell densities were examined on a Zeiss Axioplan epifluorescence microscope with an Axiocam CCD camera and relative rhodamine fluorescence intensity was analyzed by morphometric quantitation using Adobe Photoshop as described previously (17). A third portion of the tumor was minced, 0.25% trypsin digested, and dispersed in Eagle’s medium supplemented as described above. Cell cytotoxicity assays were then done with \(^{3}H\)leucine and/or \(^{3}H\)thymidine as described previously (16), and results were expressed as IC50 (concentration of DAB389EGF yielding 50% control thymidine or leucine incorporation).

Pharmacokinetic Studies. Tumor-bearing and control nude mice were injected i.t. and i.v. with 3 \( \mu \)g DAB389EGF and blood samples were obtained pretreatment and at 5, 30, 60, and 90 minutes post-treatment. Sera were isolated and saved at -80°C. Dilutions of mouse serum in medium were tested along with known dilutions of DAB389EGF in pretreatment mouse sera in medium on U373MG cells by the previously described \(^{3}H\)thymidine incorporation inhibition assay (16) and serum concentrations were calculated based on the IC50 ratios.

Immunogenicity Studies. BALB/c mice were injected s.c. with 150 \( \mu \)g/kg DAB389EGF every other day for six doses. At day 25, mice were euthanized and blood samples were collected via cardiac puncture. Blood samples from nontreated mice were used as a negative control. Sera were stored at -80°C until assayed. Anti-diphtheria toxin antibodies were assayed as described previously with the substitution of DAB389EGF for DT389GMCSF (18).

Statistical Analysis. Survival was analyzed by the Kaplan-Meier method. For comparison of tumor volumes, weights, activated caspases by immunoblot density, EGFR densities, and IC50s, a Mann-Whitney or Kruskal-Wallis test was used. Significance of differences was assumed at \( P \leq 0.05 \).

RESULTS

MTD of DAB389EGF. The MTD of DAB389EGF given i.t. was 3 \( \mu \)g every other day for 6 to 12 doses. No deaths were observed among 16 animals (Fig. 1). In contrast, animals
were observed. The DLT appeared to be renal failure. In the liver (Fig. 2). No injuries to brain, lungs, heart, or spleen.

mice showed severe renal tubular necrosis and rare apoptotic cells at day 5. Necropsies of treated moribund nude mice and BALB/c mice after 5 days receiving 5 or 10 μg doses (n = 8) had a mortality of 25% and 87%, respectively.

**DLT of DAB389EGF.** At DAB389EGF doses of ≥5 μg, animals developed listlessness, lethargy, dehydration, and hunched posture. Blood chemistries revealed elevated blood urea nitrogen and alanine transaminase (Table 1). Additional groups of five BALB/c mice given two 10 μg DAB389EGF doses s.c. every other day showed elevated creatinine (2.4 ± 1.3 versus 0.5 ± 0.08 mg/dL control; P = 0.01) and aspartate transaminase (8,232 ± 4,387 versus 1,086 ± 684 units/L control; P = 0.007) in addition to high alanine transaminase (7,327 ± 6,465 versus 137 ± 51 units/L control; P = 0.03) and blood urea nitrogen (293 ± 64 versus 18 ± 4 mg/dL control; P = 0.0004) on day 5. Necropsies of treated moribund nude mice and BALB/c mice showed severe renal tubular necrosis and rare apoptotic cells in the liver (Fig. 2). No injuries to brain, lungs, heart, or spleen were observed. The DLT appeared to be renal failure.

**Recovery from DAB389EGF-Induced Toxocities.** Three mice after 5 μg DAB389EGF treatment developed markedly reduced activity, dehydration, hunched posture, and >20% loss of body weight. These mice were given saline 0.5 mL by s.c. or i.p. injections daily for 3 or 6 days (Fig. 3). All animals recovered and regained their pretreatment weight within 10 days.

**U87MG Glioma Sensitivity to DAB389EGF.** Thymidine incorporation inhibition assays of DAB389EGF on U87MG cells before animal passage yielded IC₅₀ of 26,000, 49, 28, 8, and 7 ± 2 pmol/L with fusion protein incubation times of 12, 24, 36, 48, and 72 hours, respectively. Leucine incorporation inhibition yielded similar results with IC₅₀ of >10,000, 355, 60, 11, and 9 pmol/L with fusion protein incubation times of 12, 24, 36, 48, and 72 hours, respectively. After passage in athymic nude mice, tumors from untreated mice were harvested and reassayed by thymidine incorporation inhibition assays. With 72-hour incubation, the U87MG cells recovered from tumors had a DAB389EGF IC₅₀ of 7 pmol/L.

**U87MG Tumor Growth in Athymic Nude Mice.** Athymic nude mice (n = 26) inoculated s.c. with U87MG glioma cells showed rapid tumor growth. Mean ± SE tumor volume reached 112 ± 15 mm³ by day 7. The tumor then doubled in volume (209 ± 27 mm³) by day 12 and doubled again (620 ± 72 mm³) by day 18. The mean tumor volume continued to double with a doubling time of ~4 days until animals were sacrificed on day 29 (mean tumor volume = 3,115 ± 756 mm³). Based on these results, we could detect a 25% or 50% tumor growth inhibition with 15 or 5 animals per group, respectively, with a two-sided type I error of 5% and a power of 0.90. Pathology of the tumor confirmed the malignant histology.

**Antiglioma In vivo Efficacy of Diphtheria Fusion Proteins.** We initially explored the antiglioma activity in vivo of different doses of DAB389EGF i.t. versus saline control in cohorts of five animals (Fig. 4A). At day 30, the control tumors were 3,220 ± 760 mm³; the 1 μg dose tumors were 543 ± 261 mm³ (compared with control; P = 0.01); the 5 μg dose tumors were 176 ± 132 mm³ (compared with control; P = 0.005); and the two 10 μg dose tumors were 0 mm³. Because of the excessive toxicities of the 5 and 10 μg doses, we selected 3 μg for further study. Cohorts of 16 animals were treated with 3 μg DAB389EGF or 10 μg DAB389IL2 i.t. every other day for six doses (Fig. 4B). The DAB389EGF-treated animals showed tumor growth inhibition. On day 29, the PBS control tumors were 3,115 ± 756 mm³, the DAB389IL2-treated tumors were 1,682 ± 358 mm³ (P = 0.644 versus control), and the DAB389EGF-treated tumor were 1 ± 1 mm³ (P = 0.0001

**Fig. 1** Female athymic nude mice (ages 4-6 weeks) were inoculated with 10 million U87MG tumor cells on day 0. When tumors had reached 100 mm³ in volume (day 7), i.t. injections of DAB389EGF or DAB389IL2 were given every other day. Cohorts of eight animals were treated. One group received DAB389IL2 at 10 μg per dose on days 6-16 for a total of six doses (A). A second group received DAB389EGF at 3 μg per dose on days 6-16 (a total of 6 doses) and for two mice repeat injections on days 22-32 (a total of 12 doses; ◊). A third group received DAB389EGF at 5 μg per dose on days 6-16 and 28-34 (a total of 9 doses; ). A final group received DAB389EGF at 10 μg per dose on days 6-10 or 14 for a total of 3 to 5 doses (○).

**Table 1** Blood urea nitrogen and alanine transaminase serum levels of DAB389EGF-treated mice

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<th>Dose (μg)</th>
<th>Sampling day</th>
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NOTE: Blood urea nitrogen, normal: 8-24 mg/dL; alanine transaminase, normal: 0-36 units/L.

*Moribund animals.
versus control). By day 60, 12 of 16 (75%) DAB389EGF-treated mice remained in remission both grossly and by histopathology. Three animals had tumor recurrence and received a second course of DAB389EGF. One of these animals became disease free at day 60 both grossly and by histology. Two of the retreated animals again had tumor regrowth by day 60 of 20 and 85 mm³. One animal had no visible tumor regrowth and was not treated with a second course but showed five tumor cells in the skin by microscopic histopathology on day 60.

Properties of Relapsing Tumors. Tumors recurring after initial response to DAB389EGF were examined for EGFR and sensitivity in tissue culture to DAB389EGF. Tumors retained EGFR expression with similar rhodamine fluorescence intensity values (results not shown). Tumor cells remained sensitive to DAB389EGF with IC₅₀ of 4 ± 1 versus 7 pmol/L for control tumors. Animals with relapsing tumors after initial response to DAB389EGF were retreated. Tumors again responded (Fig. 4C).

DAB389EGF Pharmacokinetics and Immune Response. The serum levels after i.t. administration of DAB389EGF were immeasurable at all time points (<5 ng/mL). In contrast, after i.v. administration, the half-life was 30 minutes with peak level of 14 ± 7 ng/mL from a 3 μg dose. Antibodies to diphtheria toxin were absent in four of five control mice (<1 ng/mL) and at 1 μg/mL in the fifth control mouse (mean = 0.2 ± 0.4 μg/mL). In contrast, on day 25 after the 2-week treatment course, all five mice had elevated antibody levels (mean = 1028 ± 532 μg/mL). The difference from control mice was significant (Student t test P = 0.006).

DISCUSSION

The fusion protein DAB389EGF is tolerated by rodents at doses that have significant antitumor activity when given i.t. The MTD of 3 μg per mouse corresponds to 150 μg/kg and is 4-fold higher than the MTD with i.v. administration (15). The short half-life of DAB389EGF in the blood combined with a slow escape of the drug from the tumor into the vasculature produced very low blood concentrations. This may contribute to the reduced toxicity. Because other species, including humans, have similar distribution of EGFR, the results from this study should be applicable to the clinical setting. However, studies of i.t. infusions into the brain need to be done with orthotopic i.c.
rodent tumor models to confirm safety, efficacy, toxicology, pharmacokinetics, and immunology.

A complicating factor with i.t. CED is poor distribution of drug outside the tumor mass. The tumor may create an “antigenic sink” for the drug and responses may be improved by CED to postsurgical resection adjacent brain (12). Such pharmacologic considerations are poorly modeled in animals and will be repeated during clinical trials.

The target normal organ of i.t. DAB389EGF appeared to be the kidney. These results match previous observations of DAB389EGF in rats, monkeys, and patients. The presence of EGFR on renal tubule cells suggests the toxicity is ligand directed. We observed that hydration was able to reverse toxicity in a fraction of moribund animals. Such hydration may be useful in patients displaying DAB389EGF renal injury.

Repeat administration of the fusion protein produces an immune response after systemic administration to immunocompetent animals and patients. We observed anti–diphtheria toxin antibodies 4 weeks after initiation of DAB389EGF s.c. infusions in immunocompetent BALB/c mice. Thus, additional studies are needed in immunocompetent rodent glioma models to confirm efficacy of repeated courses.

The dramatic antiglioma effects observed in this study are equal to or better than those reported with other fusion proteins (8–12, 19–23). Because several of these proteins have yielded complete remissions in refractory glioma patients (4–8, 12), the results suggest that DAB389EGF will likely be useful when given by CED to such patients. This fusion protein is distinct from other clinically tested brain tumor fusion proteins both with regard to the toxin and ligand. The EGFR level in the tested U87MG human glioma cells was 65,000 per cell (16). EGFR is overexpressed on 84% of human high-grade gliomas (17). EGFR is truncated in some gliomas leading to constitutive activation (EGFRvIII), but most of the glioma cells in these patients also overexpress wild-type EGFR (24). Availability of new potent fusion proteins may overcome immune responses to these agents (25) and fusion protein–specific resistance mechanisms (26).

Further, combination therapies with different fusion proteins may yield synergistic efficacy (27, 28).

Re-treatment in our study improved the quality and duration of remissions. Because of the large size of the molecule (~45,000 Da), the drug may have limited penetration of the s.c. tumor mass; consequently, deposits of tumor may not receive significant drug and may regrow. Relapsing tumor retains EGFR and DAB389EGF sensitivity; thus, it is not surprising the tumor responds to re-treatment. Because this phenomenon may occur after CED, a re-treatment strategy may be useful clinically to consolidate response. However, as noted above, DAB389EGF is immunogenic, and methods must be developed to reduce the immunogenicity for optimal efficacy. In summary, this study provides preclinical rationale for the further development of DAB389EGF for interstitial therapy of refractory gliomas.

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