A Diphtheria Toxin-Epidermal Growth Factor Fusion Protein Is Cytotoxic to Human Glioblastoma Multiforme Cells

Tie Fu Liu, Kimberley A. Cohen, Jason G. Ramage, Mark C. Willingham, Andrew M. Thorburn, and Arthur E. Frankel

ABSTRACT

The cytotoxicity of a diphtheria toxin-human epidermal growth factor fusion protein (DAB<sub>389</sub>EGF) was tested against 14 human glioma cell lines. After cells were cultured for 48 h with various concentrations of DAB<sub>389</sub>EGF, the percentage reduction in thymidine incorporation was determined. For 13 of 14 cell lines, potent cytotoxicity was observed, with IC<sub>50</sub> values of 0.4–50 μM. The epidermal growth factor receptor (EGFR) density of these cell lines was determined by immunofluorescence microscopy, flow cytometry, and radioligand binding. These assays correlated well with each other and demonstrated EGFR levels of 15,000–230,000/cell for 13 of 14 cell lines. The cell line U138MG, which lacked EGFR, was the only cell line insensitive to DAB<sub>389</sub>EGF. Linear regression analysis showed a good correlation between EGFR density and DAB<sub>389</sub>EGF sensitivity (P < 0.001) and between results of flow cytometry and radiolabeled binding assays of EGFR density (P = 0.01). DAB<sub>389</sub>EGF may have potential for intracranial therapy of EGFR-positive glioblastomas.

INTRODUCTION

There are 20,000 cases of primary brain tumors per year in the United States (1). More than 80% of these tumors are high-grade infiltrative astrocytoma/GBM, which arise from malignant transformation of astrocytes (2). GBMs are the third leading cause of cancer-related death in adolescents and adults in the age range 15–34 years. The median survival for GBM patients is 1 year for newly diagnosed patients and 6 months for patients with recurrent disease (3). Fewer than 20% of GBM patients are alive at 2 years. Thus, this disease is associated with a very poor prognosis. Chemotherapy- and radiotherapy-resistant tumor cells and local tumor growth are the main causes of death, with 90% of recurrences at or adjacent to the site of origin of the disease (4, 5). Novel agents with different mechanisms of action are needed for these brain tumors.

Fusion proteins targeting cell surface receptors selectively overexpressed on GBMs represent one such different approach. The brain tumor-selective ligand directs the protein to the glioma cell surface; the peptide toxin is then internalized into the cell, translocates to the cytosol, and catalytically inactivates protein synthesis, leading to cell death. Several fusion proteins have been synthesized for brain tumor therapy that are reactive with high-affinity IL13 receptors, Tf receptors, urokinase receptors, and IL4 receptors. CED was used to introduce these agents into the brain tumor interstitium. This method creates a bulk flow that supplements diffusion and achieves drug concentrations orders of magnitude higher than concentrations achieved by systemic administration for large areas of the brain parenchyma. Clinical remissions lasting years were observed in many patients (6, 8). Because of the toxicities with several of the agents (e.g., T1-CRM107 and IL4[38–37]-PE38KDEL) and the lack of binding to all brain tumors with others (e.g., IL13PE38QQR), there is a need for additional brain tumor fusion proteins.

An attractive target is the EGFR. Glioma cells often express ~100,000 EGFR molecules/cell, whereas normal brain tissues (glial, neuronal, and endothelial cell lineages) display only a few thousand EGFR molecules/cell (11). Half of the glioblastomas show amplification of the EGFR gene (up to 60-fold) and overexpression of EGFR mRNA and protein (12). Increased EGFR expression is correlated with a poorer prognosis in brain tumor patients (13). Furthermore, EGFR overexpression may be critical for maintenance of the malignant phenotype because glioma cells possess an EGFR/tumor growth factor-α autocrine loop (14). This autocrine loop produces constitutive stimulation of pathways important for cell survival, spreading, and proliferation. Because the EGFR binds EGF with high affinity and the EGF-EGFR complex internalizes efficiently by receptor-mediated endocytosis, the EGFR is an excellent target for fusion proteins with toxins such as ricin, PE, and DT. On the basis of these observations, we chose to investigate the antiangioma activity of a DT-EGF fusion molecule, DAB<sub>389</sub>EGF.

DNA encoding a methionine, the catalytic and translocation domains of DT (amino acid residues 1–387), a His-Ala linker, and amino acids 1–53 of EGF was inserted into a bacterial expression plasmid under the control of a trc promoter and used to transform Escherichia coli (15). After induction with isopropyl β-D-thiogalactopyranoside, inclusion bodies were washed, denatured, and renatured. Recombinant protein was purified by anion-exchange chromatography and filter sterilization. DAB<sub>389</sub>EGF had a molecular weight of 48,522, reacted with antibodies to DT and EGF on immunoblots, and was cytotoxic at pM concentrations to the EGFR-positive A431 human vulvar carcinoma cell line. Cell killing was dependent on endosome acidification.

The fusion protein had an affinity for the EGFR that was 15–30-fold lower than for EGF itself. Rats administered DAB<sub>389</sub>EGF by daily bolus i.v. infusions for 10–14 days had a MTD of 30 μg/kg/day and a DLT of renal and hepatic injury (16). The circulating half-life was <1 min with peak levels of 20 ng/ml at doses of 40 μg/kg. [135I]Methionine-labeled DAB<sub>389</sub>EGF administered i.v. to rats distributed rapidly to the liver (61% of the injected dose) and kidney (6% of injected dose). Antibody formation to DT occurred within 1 month in all animals.

DAB<sub>389</sub>EGF was slightly more toxic to cynomolgus monkeys after i.v. infusions with an MTD of 20 μg/kg/day for 10 days. The DLT was also liver and kidney damage. Nude mice inoculated s.c. with A549 human EGFR-positive lung adenocarcinoma cells and treated starting 24 h later with DAB<sub>389</sub>EGF (25 μg/kg/day for 10 days) had a 50% tumor growth inhibition by day 17 with complete tumor regressions in 2 of 10 treated mice. Three Phase I/II clinical studies were conducted with five to nine 30-min i.v. infusions of DAB<sub>389</sub>EGF over 1–3 weeks in 72 patients with EGFR-positive metastatic carcinomas (17). Peak DAB<sub>389</sub>EGF serum levels were 0–50 ng/ml. One-fourth of the patients had high pretreatment anti-DT antibody titers; all patients had high anti-DT antibody titers 1 month posttreatment.
The MTD was 6 µg/kg/day, and the DLT was renal and liver injury. Only one 6-month partial remission in a patient with non-small cell lung carcinoma was observed.

Many of the properties of DAB$_{389}$EGF that compromised clinical activity with systemic administration should be beneficial for brain tumor CED therapy. The short half-life, circulating anti-DT antibodies, and clearance by the liver and kidneys should not influence local delivery in the brain. Normal tissue toxicities should be reduced because high concentrations of fusion protein will be present only in the brain and brain tumor. With the known clinical experience with DAB$_{389}$EGF in patients, we wanted to extend the work to a brain tumor application. In this report, we describe the sensitivity of a battery of human glioma cell lines to DAB$_{389}$EGF and correlate the sensitivity with EGFR expression. The results establish a rationale for CED DAB$_{389}$EGF therapy of recurrent EGFR-positive gliomas.

**MATERIALS AND METHODS**

DAB$_{389}$EGF. DAB$_{389}$EGF was synthesized and partially purified as described previously (16). Vials contained 500 µg of sterile-filtered, lyophilized DAB$_{389}$EGF in PBS (pH 7.2) containing 1% mannitol and 50 µM EDTA (lot no. 3E113EB2; Ligand Pharmaceuticals). Vials were stored at −80 °C. Fusion protein was prepared for experiments by adding 2 ml of sterile water and gently stirring. Dissolved fusion protein was stored at 4 °C.

**Cell Lines.** Cell lines A431, U373MG, and U118MG were obtained from the Wake Forest University Tissue Culture Core Laboratory. Cell lines A172, D87G05SM, T98G, U373MG, and U138MG were purchased from the American Type Culture Collection (Rockville, MD). Cell lines LN504, GAMG, DKMG, GMS10, 42MGBA, SNBin9, and 8MGBA were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). A431 cells were grown in DMEM containing 10% fetal bovine serum, penicillin/streptomycin, and L-glutamine. U373MG and U138MG cells were grown in Eagle’s medium supplemented with penicillin/streptomycin, L-glutamine, nonessential amino acids, 0.1 g/l sodium pyruvate, and 10% fetal bovine serum. A172 and U118MG cells were grown in Dulbecco’s medium containing 1.5 g/l sodium bicarbonate, penicillin/streptomycin, L-glutamine, and 10% fetal bovine serum. LN504 and SNBin9 cells were expanded in Dulbecco’s medium containing 10% fetal bovine serum. GAMG cells were grown in Dulbecco’s medium containing 10% fetal bovine serum, L-glutamine, and nonessential amino acids. DKMG cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and L-glutamine. GMS10 cells were grown in Dulbecco’s medium containing 10% fetal bovine serum and L-glutamine. D87G05SM cells were grown in RPMI 1640 supplemented with penicillin/streptomycin, L-glutamine, 0.1 g/l sodium pyruvate, 10% fetal bovine serum, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, sodium pyruvate, 1 mg/ml AMP, 10 mg/l adenine, 1 mg/l thymidine, 50 mg/l L-isoleucine, 100 mg/l L-cystine, 5 g/l HEPES, 50 mg/l L-proline, and 15 mg/l hypoxanthine. T98G and U87MG cells 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. 42MGBA cells were grown in 50% RPMI 1640/50% minimal essential medium with Earle’s salts plus 20% fetal bovine serum. 8MGBA cells were grown in minimal essential medium with Earle’s salts plus 20% fetal bovine serum. Cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO$_2$ in air.

**Cell Line Sensitivity to DAB$_{389}$EGF.** Aliquots of 5 × 10³ cells were incubated in triplicate in 100 µl of medium (same as that used to grow the cells) in Costar 96-well flat-bottomed plates. After 24 h, 50 µl of DAB$_{389}$EGF in medium were added to each column to yield concentrations ranging from 0 to 10,000 pM, and the cells were incubated at 37 °C in 5% CO$_2$ for another 48 h. After the incubation, 1 µCi of [H]thymidine or [H]leucine (NEN DuPont, Boston, MA) in 50 µl of medium was added to each well, and incubation was continued for an additional 16 h at 37 °C in 5% CO$_2$. Plates were frozen at −80 °C and thawed, cells lysates were harvested using a Skatron Cell Harvester (Skatron Instruments, Lier, Norway) on to glass fiber mats, and the cpm of incorporated radiolabel were counted using an LKB liquid scintillation counter gated for [H]. The IC$_{50}$ was defined as the concentration of toxin that inhibited thymidine or leucine incorporation by 50% compared with control wells. The percentage of maximum [H]thymidine or [H]leucine incorporation was plotted versus the log of the toxin concentration, and nonlinear regression with a variable-slope sigmoidal dose-response curve was generated along with IC$_{50}$ with use of GraphPad Prism software (GraphPad Software, San Diego, CA). All assays were performed in triplicate.

**Detection of EGFR by Immunofluorescence Microscopy.** Aliquots of 100,000 cells for each of the cell lines were grown overnight attached to the surface of 35-mm Costar tissue culture dishes in 1 ml of medium. Cells were washed twice with cold PBS, blocked with 1% BSA in PBS, incubated for 60 min on ice with either 10 µg/ml murine monoclonal antibody to the extracelular domain of the EGFR (31G7; Zymed Laboratories, South San Francisco, CA) or 10 µg/ml murine monoclonal antibody isotype control (08-6599; Zymed Laboratories), washed three times with PBS, incubated for 60 min on ice with 25 µg/ml affinity-purified goat antimouse immunoglobulin conjugated to rhodamine in PBS/BSA (115-025-062; Jackson ImmunoResearch, West Grove, PA), washed three times with PBS, fixed with 3.7% formaldehyde in PBS, mounted under a no. 1 coverslip in glycerol-PBS (90:10), and examined under a Zeiss Axioskop epifluorescence microscope. Photomicrographs were obtained using a Zeiss AxioCam charge-coupled device camera with a fixed magnification, exposure, and gain setting for all cell lines.

**Flow Cytometry Measurements of EGFR Density.** Two aliquots, each containing 1 × 10⁶, for each of the cell lines were pelleted at 600 × g and resuspended in 80 µl of PBS/BSA. To one of each aliquot of cells, we added 20 µl of R-phycocerythrin-conjugated mouse anti-EGFR antibody (EGFR1; BD Biosciences, Mountain View, CA) or phycocerythrin-conjugated mouse IgG2b isotype control antibody (27-35; BD Biosciences). The cells were incubated at 4 °C for 30 min, washed with PBS/BSA, and resuspended in 1 ml of PBS/BSA. We added 250 µl of 3.7% formaldehyde to each tube, and the cells were assayed on an EPICS-XL flow cytometer (Coulter, Hialeah, FL) with filters set for phycocerythrin fluorescence detection. Quantifluor beads (BD Biosciences) were used to determine the ratio of phycocerythrin fluorescence channel number of phycocerythrin molecules. The phycocerythrin conjugated used had one molecule of phycocerythrin per antibody. EGFR expression was calculated assuming single antibody molecules bound per EGFR (also referred to as ABC).

**Radiolabeled EGF Binding.** We cultured 100,000-cell aliquots in medium in wells of Costar 24-well flat-bottomed plates overnight at 37 °C in 5% CO$_2$. Wells were then washed three times with 200 µl of PBS, incubated 30 min at 37 °C in 5% CO$_2$ in 200 µl of binding buffer [RPMI 1640 plus 0.2% sodium azide plus 2.5% BSA plus 20 µg/ml HEPES (pH 7.4)] with various concentrations (1–2000 pm) of 125I-labeled EGF (195 mCi/mg; ICN, Irvine, CA) with or without unlabeled (400 µM) human EGF (236-EG; R&D Systems, Minneapolis, MN). Wells were then incubated for 1 h at 37 °C in 5% CO$_2$; medium was then collected from the wells, transferred to glass tubes, and counted. Wells were then washed three times with PBS and incubated 30 min with 300 µl of 1 M sodium hydroxide. Well contents and two 300-µl water washes were transferred to glass tubes and counted. Counts were measured in a Packard Auto-Gamma 5650 gamma counter gated for 125I with 50% counting efficiency. Background cpm were calculated by linear extrapolation from incubation with excess unlabeled EGF. Specific binding saturation curves were made using GraphPad Prism (GraphPad Software). Nonlinear regression analysis was used to calculate the Kd and Bmax of the EGFR.

**RESULTS**

**Sensitivity of Cultured Cells to DAB$_{389}$EGF.** The A431 epidermoid carcinoma cell line was sensitive to DAB$_{389}$EGF. The mean (±SD) IC$_{50}$ for proliferation inhibition (thymidine incorporation) after 48 h of incubation was 0.9 ± 0.7 pm. Similar sensitivity was observed with 13 of 14 glioma cell lines (see Table 1). The cytotoxicity assays yielded reproducible IC$_{50}$ with ranges <30% from the mean (Fig. 1). The potency was high for the 13 sensitive cell lines, ranging from 0.4 to 50 pm. The cell line with low or absent EGFR expression (U138MG) showed no sensitivity to DAB$_{389}$EGF. Protein synthesis inhibition by DAB$_{389}$EGF was measured in U373MG and U87MG cells and yielded IC$_{50}$ within 50% of the proliferation inhibition IC$_{50}$. The protein synthesis inhibition IC$_{50}$ were 1.2 pm and 2.1 pm,

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DAB389 EGF was potently cytotoxic to all glioblastoma cell lines with EGFR overexpression. Interestingly, DAB389 EGF showed toxicity to glioblastoma cells similar to that of other fusion proteins, including Tf-CRM107 (6), IL4[38–37]-PE38KDEL (7), IL13PE38QQR (8), DTIL13 (10), and 425.3-PE (18), and slightly greater toxicity than DTAT (9). The greater potency of the EGFR-, TF receptor-, IL4 receptor-, and IL13 receptor-targeted toxins on gliomas may relate to the presence of higher numbers of receptors, more rapid internalization, or more efficient translocation from intracellular compartments than the urokinase receptor-targeted molecule. Urokinase receptor is linked to the plasma membrane by glucosyl phosphatidylinositol and may be less efficiently internalized.

### DISCUSSION

DAB389 EGF was potently cytotoxic to all glioblastoma cell lines with EGFR overexpression. Interestingly, DAB389 EGF showed toxicity to glioblastoma cells similar to that of other fusion proteins, including Tf-CRM107 (6), IL4[38–37]-PE38KDEL (7), IL13PE38QQR (8), DTIL13 (10), and 425.3-PE (18), and slightly greater toxicity than DTAT (9). The greater potency of the EGFR-, TF receptor-, IL4 receptor-, and IL13 receptor-targeted toxins on gliomas may relate to the presence of higher numbers of receptors, more rapid internalization, or more efficient translocation from intracellular compartments than the urokinase receptor-targeted molecule. Urokinase receptor is linked to the plasma membrane by glucosyl phosphatidylinositol and may be less efficiently internalized.

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**Table 1.** *DAB389 EGF sensitivity and EGFR content of human glioma cell lines*

<table>
<thead>
<tr>
<th>Cell line</th>
<th><em>DAB389 EGF</em> IC₅₀ (pM)</th>
<th>EGFR* (molecules/cell)</th>
<th>EGFR (Bₘₐₓ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>0.9 ± 0.7</td>
<td>215,000</td>
<td>149,000</td>
</tr>
<tr>
<td>U373MG</td>
<td>0.4 ± 0.1</td>
<td>34,000</td>
<td>86,000</td>
</tr>
<tr>
<td>U87MG</td>
<td>1 ± 0.5</td>
<td>29,000</td>
<td>65,000</td>
</tr>
<tr>
<td>42MGBA</td>
<td>3.1 ± 0.2</td>
<td>38,000</td>
<td>116,000</td>
</tr>
<tr>
<td>SNB19</td>
<td>4 ± 0.2</td>
<td>19,000</td>
<td>17,000</td>
</tr>
<tr>
<td>8MGBA</td>
<td>4.4 ± 1.3</td>
<td>17,000</td>
<td>29,000</td>
</tr>
<tr>
<td>GAMG</td>
<td>6.0 ± 3</td>
<td>81,000</td>
<td>230,000</td>
</tr>
<tr>
<td>LN405</td>
<td>10 ± 1</td>
<td>105,000</td>
<td>59,000</td>
</tr>
<tr>
<td>T98G</td>
<td>12 ± 3</td>
<td>91,000</td>
<td>110,000</td>
</tr>
<tr>
<td>DKGK</td>
<td>14 ± 3</td>
<td>34,000</td>
<td>26,000</td>
</tr>
<tr>
<td>GMS10</td>
<td>18 ± 4</td>
<td>34,000</td>
<td>26,000</td>
</tr>
<tr>
<td>A172</td>
<td>20 ± 5</td>
<td>52,000</td>
<td>42,000</td>
</tr>
<tr>
<td>U183MG</td>
<td>20 ± 12</td>
<td>28,000</td>
<td>30,000</td>
</tr>
<tr>
<td>DTRG05SM</td>
<td>50 ± 10</td>
<td>81,000</td>
<td>15,000</td>
</tr>
<tr>
<td>U138MG</td>
<td>&gt;150</td>
<td>1,100</td>
<td>550</td>
</tr>
</tbody>
</table>

* ABC represents antibody molecules/cell based on one phycoerythrin molecule per antibody and Quantibrite beads with 78,000 phycoerythrin molecules standardized with each run on the flow cytometer.

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**Correlation of DAB389 EGF IC₅₀, Radiolabeled EGF Binding, and EGFR Flow Cytometry.** There was a strong negative correlation between EGFR determined by radiolabeled binding and the DAB389 EGF thymidine incorporation inhibition IC₅₀ based on linear regression (Fig. 3A; P < 0.001). Confirming the results of the EGFR estimation, the EGFR density measured by flow cytometry and radiolabeled binding showed a positive correlation by linear regression (P = 0.01; Fig. 3B).
measures of EGFR may be useful in the design of clinical trials with DAB_{389}EGF. Patients with high-EGFR-expressing brain tumors may be more likely to respond.

This study provides some preclinical rationale for the further development of DAB_{389}EGF for therapy of refractory gliomas. The availability of clinical grade reagent and the extensive previous preclinical and clinical pharmacology and toxicology information with this drug should lead to its more rapid development (16). Although several fusion proteins are being tested for interstitial therapy of gliomas at present, the therapeutic window has been reduced because of tumor cell heterogeneity and reactivity with normal brain tissues. Thus, there remains a strong need for novel agents that are active and safe in patients with this disease.

Fig. 3. A scatterplot of DAB_{389}EGF IC_{50} and EGFR B_{max}. Linear regression yielded P < 0.0001. B, scatterplot of EGFR derived from radioligand binding (B_{max}) and flow cytometry (ABC). Linear regression yielded P = 0.01.

The binding affinity of radiolabeled EGF for the EGFR on the glioblastoma cell lines (K_{d} = 1 nm) and the receptor density/cell (B_{max} = 20,000–200,000) matched the results of previous studies of EGFR on glioblastoma cell lines (19). The high level of EGFR may occur as part of the selection pressure for establishment of glioblastoma cell lines. However, as described above, EGFR overexpression is common in high-grade gliomas, associated with a worse prognosis, and may be part of the oncogenic process leading to tumor cell proliferation (12–14).

EGFR density correlated with DAB_{389}EGF sensitivity, although because most glioma cell lines overexpressed EGFR, there were limited data with low to intermediate EGFR densities. The correlation was expected because DAB_{389}EGF must bind EGFR before cell intoxication (15). Although some correlation may be expected, it was important to test the hypothesis for several reasons. First, receptor number has not always correlated with fusion protein sensitivity. For complex heterodimer or heterotrimeric receptors, such as the IL2 receptor, the numbers of low-affinity receptors did not correlate with fusion protein sensitivity (16). In other cases, such as the granulocyte-macrophage colony-stimulating factor receptor, only low receptor numbers may be needed for DT fusion protein intoxication, and additional cell surface receptors did not significantly modify sensitivity (16). Second, EGFR mutations are frequent in gliomas and may be associated with altered ligand binding or internalization (20). Thus, we needed to obtain some information on the correlation of DAB_{389}EGF sensitivity and EGFR density even for a set of glioma cell lines. Although not necessarily reflecting the behavior of patient glioma cells in vivo, our observation of a correlation between sensitivity and receptor density among cell lines suggests that some mea-

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14. Tang, P., Steck, P. A., and Yung, W. K. The autocrine loop of TGF-alpha/EGFR and HGF/c-Met in gliomas at present, the therapeutic window has been reduced because of tumor cell heterogeneity and reactivity with normal brain tissues. Thus, there remains a strong need for novel agents that are active and safe in patients with this disease.

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