Transforming Growth Factor-α—Pseudomonas Exotoxin Fusion Protein
(TGF-α-PE38) Treatment of Subcutaneous and Intracranial Human Glioma and Medulloblastoma Xenografts in Athymic Mice

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ABSTRACT

Epidermal growth factor receptor (EGFR) is amplified or overexpressed in many malignant gliomas and other primary brain tumors but is low or undetectable in normal brain. In the present study, this differential expression has been exploited for targeted brain tumor therapy using a TGF-α-Pseudomonas exotoxin recombinant toxin, TGF-α-PE38. In vitro experiments demonstrate that the cytotoxicity of this fusion protein is primarily determined by tumor EGFR expression and that TGF-α-PE38 cytotoxicity is abolished by pretreatment with excess epidermal growth factor. Treatment with i.p. TGF-α-PE38 in nude mice bearing glioblastoma or medulloblastoma s.c. xenografts produced tumor regression and growth delay. For intracranial xenograft implants treated with i.p. TGF-α-PE38, significant increases in median survival were noted only for tumors with the highest EGFR expression. However, intracranial tumors treated with a single intratumoral injection of TGF-α-PE38 showed increased survival in all xenografts tested. These results indicate that TGF-α-PE38 is active against primary human brain tumors ranging from moderate to high EGFR expression. For intracranial tumors, however, the higher survival rates produced by intracranial injection of TGF-α-PE38 than by continuous i.p. administration suggest that increased drug clearance or impaired drug delivery reduces the efficacy of systemic TGF-α-PE38. Direct delivery of TGF-α-PE38 into brain tumors by controlled-release biodegradable polymers or intratumoral implanted catheters, or intrathecal administration into the colony stimulating factor of patients with leptomeningeal metastasis, may represent clinically useful applications of recombinant toxin therapy in tumors with high EGFR expression.

INTRODUCTION

Anaplastic astrocytoma and glioblastoma, the most common primary brain tumors in adults, respond poorly to all current therapies: median survival for patients with these tumors ranges from 19 to 67 weeks (1). Local tumor recurrence also constitutes a significant problem in medulloblastoma, the most common childhood brain tumor. Despite 5-year survivals for medulloblastoma exceeding 80% in some studies (2), nearly half of these patients will eventually die from progressive tumor. Treatment failure in patients with brain tumors is a multifactorial process involving the intrinsic resistance of these tumors to radiation therapy and chemotherapy, the development of acquired treatment resistance, and limitations to drug delivery due to poor tumor vascularity or blood-brain barrier restrictions (3, 4). Local recurrence of brain tumors represents the most common pattern of treatment failure (5). Accordingly, the identification of new therapeutic agents that have high intrinsic activity against brain tumors and are appropriate for local therapy remains a major goal.

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2 The abbreviations used are: TGF-α, transforming growth factor-α; EGFR, epidermal growth factor receptor; mAb, monoclonal antibody; PE, Pseudomonas exotoxin; i.c., intracranial; EGF, epidermal growth factor; ID90, drug concentration that results in a 10% survival of colony forming units.

TGF-α1-PE represent a growing class of recombinant toxins designed for use in targeted cancer therapy. These genetically engineered chimeric proteins consist of a targeting moiety and a cytotoxic moiety. TGF-α-PE are constructed by replacing the Mr 23,000 binding domain (domain 1) of Pseudomonas exotoxin with transforming growth factor-α (6). Additional site-specific mutations in Pseudomonas exotoxin result in greater chemical stability and have been designated TGF-α-PE38 (amino acids 253–334 and 381–613), PE40 (amino acids 253–613 of PE), and others (7). These chimeric proteins are extremely toxic to tumor cells that have a relatively high expression of EGFR (e.g., lung, breast, and bladder carcinomas). The high concentration of these receptors in human liver, however, results in dose-limiting hepatotoxicity when TGF-α-PE38 is administered by systemic (i.e., i.v. or i.p.) routes. Many brain tumors have high EGFR expression, whereas the density of these receptors in normal brain is relatively low (8). Amplification of the erb-B oncogene is found in up to 50% of human glioblastomas, and other primary brain tumors, including ependymomas and menigioma, have also been shown to overexpress EGFR (9). Therefore, cytotoxic agents targeted to EGFR should have a high therapeutic index for the treatment of both primary brain tumors and tumors with high EGFR density that metastasize to brain (10).

The present study was designed to evaluate the cytotoxic activity in vitro and in vivo of TGF-α-PE38 against human glioblastoma and medulloblastoma cell lines that overexpressed EGFR to different levels. We compared the effect of i.p. and i.c. administration of the toxin on tumor xenografts implanted s.c. or intracranially in nude mice. The results suggest that restrictions of TGF-α-PE38 blood-to-tumor transfer which reduce the effectiveness of systemic (i.p.) drug administration may be overcome by local delivery methods. Development of TGF-α-PE38 may provide an effective means to treat brain tumors, particularly for glioblastomas that are notoriously refractory to other therapies.

MATERIALS AND METHODS

Cell Lines. Human brain tumor cell lines derived from adult patients with glioblastoma (U87, U251, U373, WF, JHG30, and JHG31), from a child with glioblastoma (JHG10), and from a child with medulloblastoma (DAOY) were used in these studies. U87 and U373, which are established and continuous glioblastoma cell lines, were obtained from American Type Culture Collection (Rockville, MD). U251 glioblastoma was kindly supplied by Henry Friedman (Duke University, Durham, NC). WF, a continuous glioblastoma cell line, was the gift of Joan Shapiro (Barrows Neurological Institute, Phoenix, AZ). JHG30 and JHG31 were derived at The Johns Hopkins Hospital from adult patients with glioblastoma and have been maintained in continuous culture for more than 70 serial passages. JHG10, derived at The Johns Hopkins Hospital from a 7-year-old boy with glioblastoma, has been maintained in continuous culture for more than 60 passages. Immunohistochemical studies were uniformly positive for glial fibrillary acidic protein and the karyotypic analysis was as...
follows: 96, XXY, +1, -6, -9, -10, -11, -12, -13, -14, +16, +19, +20, +22p, -3q, +4, -7q, -7q, -10q, + marker. Double minute chromosomes were present in the majority of the karyotypes analyzed from this cell line; however, EGFR, c-myc, and H-ras were not amplified or overexpressed. DAOY medulloblastoma was kindly supplied by Henry Friedman (Duke University); its establishment and phenotypic and molecular characterization have been described (11–13). The A431 human epidermoid carcinoma cell line which overexpresses EGF receptor (14) is sensitive to TGF-α-PE fusion proteins (15) was used as a control. All cell lines were grown in Rich ter's zinc-oxide medium supplemented with 20% fetal calf serum. Cultures were maintained under aseptic conditions and incubated at 37°C in humidified 5% CO₂.

Recombinant Toxins. Chimeric proteins were constructed by the fusion of the growth factor TGF-α with domains 2 and 3 of the Pseudomonas exotoxin. Three TGF-α-PE recombinants were used in this study. TGF-α-PE38 denotes the site-specific mutations in Pseudomonas exotoxin domain 2 by deletion of amino acids 335–380. TGF-α-PE40 includes the complete amino acid sequence 253–613. TGF-α-TP40 denotes cysteine to serine substitutions at Pseudomonas exotoxin amino acids 265 and 287.

Cytotoxicity Assays. Clonogenic assays of drug cytotoxicity were performed with cells in log phase. For each experiment, cells were harvested by trypsinization, washed with Hank's balanced salt solution, and resuspended in medium containing 20% fetal calf serum. Cells in fresh medium were pipetted into 35-mm Petri dishes and incubated at 37°C in 5% CO₂ for 24 h to permit attachment. The medium was removed and replaced with medium containing filter-sterilized drug. Cytotoxicity at each drug dilution was assayed in triplicate. Dishes were incubated for 6–12 days at 37°C in 5% CO₂, washed free of medium, and stained with methylene blue, and colonies containing >50 cells were counted.

EGF Receptor Western Blots. Log-phase cells were harvested with 0.25% trypsin in calcium- and magnesium-free phosphate buffered saline containing 2 mM EDTA, then washed twice in Hank's balanced salt solution and centrifuged briefly to form a cell pellet. The pellet (5 × 10⁷) cells were resuspended in a 2× phosphate-buffered saline extraction buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium deoxycholate, 1 mM EDTA, 10 μg/ml leupeptin, and 10 μg/ml chymotatin and incubated on ice for 15 min. The cell suspension was centrifuged at 1000 × g for 5 min, supernatant fraction was collected, and the protein concentration was quantified by a spectrophotometric assay (16).

Polyacrylamide gel electrophoresis was performed as described by Laemmli (17). Polyacrylamide gels 0.75 mm thick consisted of a 14-cm-high 7.5% (w/v) acrylamide resolving gel and a 1.5-cm-high 5% (w/v) stacking gel. Adjacent wells were loaded for equal protein content. After electrophoretic separation, the proteins were transferred to nitrocellulose. Nonspecific protein binding sites were blocked with 5% nonfat dry milk in a Tris-saline buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4) that contained 0.2% Tween 20. A monoclonal antiepidermal growth factor receptor antibody raised against A431 carcinoma cells (Sigma Chemical Co., St. Louis, MO) was used as the primary antibody. Secondary antibody consisted of biotinylated goat anti-mouse Ig (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Immunoblotting was performed by the electrogenerated chemiluminescence method according to the manufacturer's instructions (Amersham, Arlington Heights, IL).

Animals. Female athymic nude mice (Crl nu/nu BR), 3 weeks of age or older, were purchased from Charles River Laboratories (Wilmington, MA). Nude mice were housed in filter-top cages in a separate facility and maintained in an aseptic environment with laminar flow filtered ventilation.

Xenograft Tumor Lines. U-251 and U-373 glioblastoma, DAOY medulloblastoma, and A431 carcinoma were grown as xenograft tumors in athymic mice. All xenograft tumors were harvested from nude mouse body cultures. Log-phase cells were harvested by trypsinization, washed, and centrifuged into a pellet. The cell pellet was resuspended in sterile 0.9% saline, and ≥2 × 10⁶ viable cells were injected s.c. into the flank of a nude mouse.

Tumors s.c. All experiments were performed with tumors that had undergone at least 10 serial passages as xenograft tumor in the flanks of nude mice. Stock animals bearing tumor were sacrificed by cervical dislocation, and the flank xenograft tumor was dissected free of cutaneous and connective tissue and external vascularule in a sterile laminar flow hood. The tumors were coarsely minced, then passed through a 20-mesh screen in a tissue press. The xenograft homogenate was passed sequentially through a 20- and 22-gauge needle, and injected into the barrel of a sterile 500-μl Hamilton syringe. Then 50 μl of tumor homogenate were injected s.c. through a 22-gauge needle into the right flank of each nude mouse. The length and width (mm) of s.c. xenograft tumors were measured every 2–3 days with vernier calipers (Monostat Corp., New York, NY). Tumor volume was calculated by the following formula (18):

$$\text{volume} = \frac{\text{width}^2 \times \text{length}}{2}$$

Intracranial Tumor Transplantation. Xenograft tumors for intracranial implantation were harvested as described above, except that tumors were sequentially processed through a 20-mesh screen in a tissue press, then pressed through a 60-mesh cytoseive screen. Dissociated cells were washed through the cytoseive with RPMI 1640 and centrifuged briefly to form a pellet, which was then resuspended in an equal volume of Matrigel (Collaborative Biomedical Products, Bedford, MA) and injected into the barrel of a 25-μl Hamilton syringe. Recipient animals were anesthetized by injection with sodium pentobarbital (40 mg/kg), and 20 μl of tumor suspension were injected to a depth of 4.5 mm into the right cerebral hemisphere site with the aid of a plastic template. Animals were examined 2–3 times each week for the first 2 weeks, then daily, for evidence of neurological abnormality, weight loss, or death.

PE38 Toxicity. The maximum tolerated doses of TGF-α-PE38 for administration of drug by the i.p. or i.c. route were determined separately. For i.p. toxicity studies, cohorts of 4 mice each were treated with TGF-α-PE38 at doses of 50, 75, or 100 μg/kg/24 h administered by miniosmotic pump (see below). For intracranial toxicity studies, cohorts of 5 or 6 mice each were treated with TGF-α-PE38 at doses of 0.1, 0.5, 1.0, or 5.0 μg in 20-μl injection volumes. For both studies, weight was measured daily and the appearance of abnormal neurological signs was also monitored daily. Animals were observed for 21 days after treatment and the date of death was recorded.

Xenograft Treatment s.c. with TGF-α-PE38 Administered by i.p. Osmotic Pump. Nude mice bearing s.c. xenograft tumors were treated when the median tumor volume was larger than 400 mm³ for brain tumor xenografts or 300 mm³ for A431. The pharmacokinetic characteristics and therapeutic efficacy of continuous i.p. administration of TGF-α-PE38 have been described by Pai et al. (15) and constitute the rationale for its use in these studies. Alzet miniosmotic pumps (model 2007), designed to deliver 0.5 μl/h for 7 days, were purchased from ALZA Corp. (Palo Alto, CA). At the time of surgical implantation, the pumps were filled with a filter-sterilized solution of PE38 diluted in 0.2% human serum albumin in 0.09% sodium chloride. For i.p. implantation, female athymic mice were anesthetized by injection with sodium pentobarbital (40 mg/kg). Under aseptic conditions in a laminar flow hood, the abdominal skin was cleaned with Betadine (Purdue Frederic, Norwalk, CT) and then 70% ethanol. A midline abdominal incision was made with sterile scissors, and a miniosmotic pump containing PE38 was inserted into the peritoneal cavity. The peritoneal and muscular layers were approximated with 5–0 absorbable sutures. The skin layer was closed separately with clips. In studies of the stability of TGF-α-Pseudomonas exotoxin fusion proteins, there was no evidence of degradation products or loss of activity when solutions were incubated at 37°C for more than 7 days (15).

Xenograft tumors s.c. were measured every 2–3 days with vernier calipers as described above, and the number of days for tumor volume to reach 5 times the volume at initial treatment was recorded for each animal. Treatment response was analyzed by the difference in median times between the control and PE38-treated groups to reach 5 times Thalidomide tumor volume, and by group differences in the incidence of tumor regression. Statistical significance between groups was estimated by Mann-Whitney U nonparametric tests.

Intracranial Tumor Treatment by Intratumoral PE38 Injection. Nude mice bearing intracranial xenograft tumors were treated on day 14 after tumor implantation. Mice were anesthetized with sodium pentobarbital as described above. Under aseptic conditions in a laminar flow hood, a cerebral hemisphere injection site 2 mm to the right of midline and 3 mm anterior to the external auditory canal was identified with the aid of a plastic template and marked. The scalp skin was cleaned with betadine and 70% ethanol. Intracranial injections were performed by modifications of the method described by Friedman et al. (19). TGF-α-PE38 was diluted in 0.2% human serum albumin in 0.09% sodium chloride and 3% sterile methylcellulose; 20 μl of this solution were injected by means of a 100-μl Hamilton syringe (Hamilton Co., Reno, NV) and a 27-gauge needle equipped with a plastic collar which restricted the depth of
injection to 4.5 mm. Control animals bearing intracranial tumors were treated with a single intracranial-intratumoral injection of 0.2% human serum albumin in 0.09% sodium chloride and 3% sterile methylcellulose. Mortality from this procedure was less than 5%. Response in intracranial xenografts was assessed as the difference between median survival times for control and PE38-treated animals. Statistical significance in median survival between groups was estimated by Mann-Whitney U nonparametric tests.

**RESULTS**

**EGF Receptor Expression.** To compare the expression of EGF receptor, human brain tumor cell lines were analyzed by Western blots performed with monoclonal antibodies to the extracellular or intracellular domains of the EGF receptor. EGF receptor protein was detected in all cell lines, with the highest expression in U251 and U373 gliomas and DAOY medulloblastoma, and the lowest in U87 glioma and JHG10 pediatric glioma (Fig. 1). A431 carcinoma showed a higher expression of EGF receptor than all brain tumor cell lines.

**In Vitro Cytotoxicity.** The results of clonogenic assays of adherent brain tumor cell lines and A431 carcinoma cells treated with TGF-α-PE38 or TGF-α-TP40 are presented in Table 1. Comparison of drug concentration that results in a 50% survival of colony forming units and ID_{50} values for these recombinant proteins shows PE38 to be 7- to 10-fold more cytotoxic than TP40. This finding may be due to the presence of a disulfide bond between cysteine 265 and 287 which is missing in TP40. The rank order of cytotoxicity to PE38 and TP40 was directly proportional to the expression of EGFR. Four of 7 brain tumor lines (i.e., U373, U251, DAOY, and JHG31) were highly sensitive to PE38 and have ID_{50} concentrations less than 0.3 ng/ml. Although there was no significant difference in ID_{50} or drug concentration that results in a 50% survival of colony forming units doses for PE38 within this group of sensitive brain tumor cell lines, all of them demonstrated greater cytotoxicity to PE38 and TP40 than did WF, U87, or JHG10 gliomas.

To evaluate the role of EGFR receptor binding in the cytotoxicity of PE38, DAOY cells were pretreated and coincubated with EGF concentrations that were 100-fold (w/v) in excess of the PE38 concentrations used in clonogenic assays. Fig. 2 demonstrates that competitive inhibition of TGF-α binding to EGF receptors by excess EGF eliminates the cytotoxic effect of PE38 on DAOY medulloblastoma and appears to stimulate growth.

**Toxicity.** The maximum tolerated dose (10% lethal dose) of PE38 administered by i.p. osmotic pump was estimated in groups of 4 mice at each dose. Athymic mice were treated with TGF-α-PE38 at doses calculated to deliver 50, 75, or 100 μg/kg/24 h for at least 7 days. The selection of doses was based on the similarity in cytotoxicity and chemical stability between PE38 and PE40 and the identification of the maximal tolerated i.p. pump dose of 65 μg/kg/24 h for TGF-α-PE40 in athymic mice (15). No deaths occurred at the 50- or 75-μg/kg/24 h dose during a 14-day period of observation. Two of 4 mice treated at 100 μg/kg/24 h died (days 4 and 7). To evaluate further the toxicity of TP38 administered by i.p. osmotic pump, 10 additional mice were treated with TGF-α-PE38 at the 75 μg/kg/24 h dose over 7 days. One of the mice at this dose died (day 6) and all mice showed a >10% weight loss during the first 7 days. Accordingly, the 75-μg/kg/24 h dose was selected for subsequent s.c. and i.e. xenograft experiments.

<table>
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<tr>
<th>Tumor histology</th>
<th>PE-38 (ng/ml)</th>
<th>PE-40 (ng/ml)</th>
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<tr>
<td>U251 Glioblastoma</td>
<td>0.06</td>
<td>0.28</td>
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<tr>
<td>DAOY Medulloblastoma</td>
<td>0.11</td>
<td>0.30</td>
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<tr>
<td>WF Glioblastoma</td>
<td>0.07</td>
<td>0.54</td>
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<tr>
<td>U87 Glioblastoma</td>
<td>0.54</td>
<td>1.60</td>
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<tr>
<td>JHG-10 Pediatric glioblastoma</td>
<td>0.67</td>
<td>2.00</td>
</tr>
<tr>
<td>A431 Epidermoid carcinoma</td>
<td>0.002</td>
<td>0.006</td>
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Table 1 In vitro sensitivity of human brain tumor cell lines to TGF-α-Pseudomonas exotoxin fusion proteins

*ID_{50}, drug concentration that results in a 50% survival of colony forming units.

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4 I. Pastan, unpublished observations.
To determine the maximum tolerated intracerebral dose in nude mice, groups of 5 or 6 mice were treated with 0.1, 1.0, 5.0, or 10.0 μg of TGF-α-PE38 as a single 20-μl i.c. dose. None of the mice treated with 0.1 or 1.0 μg of TGF-α-PE38 developed neurological abnormalities or died; 0 of 5 treated at the 0.1-μg dose and 2 of 6 treated at the 1.0-μg dose lost more than 10% of pretreatment weight. One of 6 mice treated at 5.0 μg and all 5 treated at 10.0 μg died within 5 days of treatment. Therefore, the 5.0-μg dose was used for subsequent i.c. xenograft experiments.

**Tumor Treatment s.c.** The response of nude mice bearing s.c. flank xenograft brain tumors to i.p. continuous infusion (osmotic pump) PE38 is illustrated in Fig. 3. No tumor regressions were observed in control animals. All tumor lines showed significant growth delay, and regression was noted in the majority of s.c. xenograft tumors (Table 2). Whereas tumor growth delay or regression persisted beyond the 7-day period of i.p. drug infusion, no complete regressions were observed. The response of s.c. tumors to i.p. PE38 parallels tumor EGFR expression in the primary brain tumor cell lines from which the xenografts were derived.

**Intracranial Tumor Treatment.** The response of nude mice bearing intracranial xenograft tumors to i.p. continuous infusion (osmotic pump) PE38 is illustrated in Fig. 4 and summarized in Table 3. All control animals developed severe neurological symptoms or died within 50 days of i.c. tumor injection; the presence of tumor was confirmed by direct inspection of the brain after i.p. injection with trypan blue. Two early deaths due to drug toxicity were observed in i.p. PE38-treated mice bearing U251 and DAOY, respectively. Intraperitoneal continuous infusion of PE38 prolonged survival for animals bearing i.c. A431 epidermoid carcinoma (P < 0.006). For tumors with a lower EGFR expression, i.p. PE38 increased survival for U251 glioma that approached significance. However, survival was not significantly increased for DAOY medulloblastoma. The response of nude mice bearing i.c. tumors to a single intratumoral injection of PE38 is illustrated in Fig. 5 and summarized in Table 3. In contrast to i.p. PE38 treatment, intratumoral injections resulted in significant increases in median survival for U251 and DAOY. The greatest increase in median survival was observed in A431 epidermoid carcinoma (73.9% increase in median survival, P = 0.0013), where one mouse survived more than 98 days. This prolonged survivor cannot be considered a cure, because examination of the brain after elective sacrifice (day 100) revealed a 2-mm-diameter tumor.

**DISCUSSION**

Strategies for the development of new treatments for brain tumors emphasize 3 factors: (a) the need to achieve local tumor control; (b) tumor-specific cytotoxicity; and (c) absence of cross-resistance with other effective therapies. Currently, the objective of reducing local tumor burden is rarely achieved by chemotherapeutic agents in anaplastic astrocytomas and glioblastomas where the response rates are often less than 30% for most agents (1). Moreover, although objective

<table>
<thead>
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<th>Table 2 Response of s.c. human brain tumor xenografts to i.p. PE38</th>
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<tr>
<td><strong>Tumor</strong></td>
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<td>U373 glioblastoma</td>
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<td>U251 glioblastoma</td>
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<td>DAOY medulloblastoma</td>
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* a Difference in median number of days from initial treatment to achieve a 5-fold increase in tumor volume in treated versus control mice.
* b Mean tumor volume measured 11-14 days after start of treatment in treated versus control mice.
* c Number of mice showing a >10% decrease in tumor volume on 2 or more separate determinations.
Fig. 4. Groups of 10 athymic nude mice were treated 14 days after intracranial implantation of human tumor xenografts with 10% of the lethal dose of TGF-α-PE38. Drug was administered continuously for 7 days by osmotic pump implanted in the peritoneal cavity. Ten controls were treated on day 14 with a 0.1% albumin solution administered in an identical fashion. Significant prolongation of survival was seen only in A431 carcinoma but not in tumors with lower EGFR expression.

EGF Receptor Targeted Therapy. EGF receptor is frequently overexpressed in glioblastoma and other brain tumors and therefore provides an appropriate target for tumor-specific therapies. Amplification of the EGFR gene has been identified in 15–50% of glioblastomas but is less common in anaplastic astrocytomas and low grade astrocytomas (27–30). Amplification or overexpression of EGFR has also been identified in oligodendroglioma, ependymoma, and meningioma (8, 28, 31). By contrast, EGF receptors are few or undetectable in normal human glial cells and neurons (8, 32), suggesting that EGF-targeted local brain tumor therapy should enjoy a relatively high therapeutic index.

Preclinical and clinical studies of anti-EGFR monoclonal antibodies provide a strong rationale for using targeted therapy for malignant gliomas. Murine IgG2a mAb 425 binds to the EGFR without stimulating tyrosine kinase activity or other signal transduction pathways. The receptor-mAb complex is then internalized and fragments of mAb 425 can be identified within the nucleus (33, 34). In human glioma xenografts, whereas unconjugated mAb 425 is able to inhibit growth, cytotoxicity is enhanced when mAb 425 is coupled with 131I or 125I (35). Objective evidence of glioblastoma tumor regression has been reported in clinical trials with 125I-labeled mAb 425 (36). Monoclonal antibodies have also been conjugated to cytotoxic drugs (21) or plant, bacterial, or fungal toxins in order to augment their cytotoxic effect (9).

Recombinant Toxins. Despite the efficacy of immunotoxins, genetically engineered recombinant toxins have several advantages over immunotoxins for clinical application (37). (a) Large amounts of recombinant toxins can be produced easily by *Escherichia coli* expression systems and purified to near-homogeneity. (b) The protein product and its binding characteristics are uniform and are not affected by chemical derivitization. (c) Recombinant toxins are more easily modified to increase chemical stability. (d) Recombinant toxins may appear to be more effective in producing significant or complete regressions in xenograft tumor models (15, 38).

TGF-α-PE is one of a growing class of recombinant toxins designed for EGF targeted therapy (6, 39, 40). The steps involved in TGF-α-PE-mediated cytotoxicity include: (a) binding of the TGF-α-toxin complex to EGFR; (b) translocation of the entire complex to the cytosol in clathrin-coated vesicles; (c) cleavage of the toxin into the translocation domain (domain II) and the protein inhibition domain (domain III); and (d) ADP-ribosylation of elongation factor 2 by domain III (41). In the absence of significant EGFR expression, TGF-α-PE has little or no cytotoxic effect (39). *In vitro* and *in vivo* studies demonstrated significant cytotoxic activity of TGF-α-PE against human epidermoid carcinoma, prostate carcinoma, and other solid tumor cell lines (15, 40), however, the activity of this class of recombinant toxin against human brain tumors has not previously been evaluated.

In the present study, *in vitro* experiments established that the cytotoxic activity of TGF-α-PE is directly related to EGF receptor expression, and that saturation of these receptors by excess EGF completely
TGF-α-PE38 TREATMENT OF BRAIN TUMOR XENOGRAFTS

Results from in vivo studies permit 3 conclusions. (a) TGF-α-PE38 is active against brain tumor xenografts that express a range of EGFR density. Tumor growth was delayed in all s.c. xenografts compared with controls, and tumor regressions were measured in the majority of treated animals. Although no significant differences in growth delay were found within the treatment groups, the median treated-control values for U373 versus DAOY approached statistical significance (P = 0.055). This result, together with in vitro cytotoxicity data (Table 1), suggest that tumors with higher EGFR expression have a greater response to TGF-α-PE38. (b) The mode of drug delivery appears to have a significant effect on TGF-α-PE38 activity in intracranial xenograft tumors. TGF-α-PE38 administered by i.p. pump was only marginally effective in brain tumor i.c. xenografts with high EGFR expression, and no significant increase in median survival was observed in DAOY medulloblastoma, which has lower EGFR expression. Only A431, which has at least 5-fold higher EGFR expression than that observed in any brain tumor xenograft, had a significant increase in median survival. (c) A single intratumoral injection of TGF-α-PE38 was more effective in increasing median survival than was continuous i.p. administration. This finding also suggests that restriction of drug delivery has important effects on cytotoxic response for macromolecules such as TGF-α-PE38.

Our results may underestimate the true response rate of intracranial tumors to intratumoral TGF-α-PE38 because of the method of administration. Although stereotaxic i.c. injection of drug, with use of the same coordinates as those for tumor injection, increases the probability that the drug will be delivered within the tumor bed itself, in our experience, the freehand injection technique causes less morbidity and mortality in nude mice than stereotaxic techniques. Nevertheless, it is likely that some injections either missed the tumor or were delivered directly into the lateral ventricle. This possibility is supported by the survival curves of nude mice treated by intratumoral injection in which 20–40% of mice had survival durations nearly identical to controls, whereas prolonged survival “tails” were observed in a similar percentage.

Nude mice treated by intratumoral injection tolerated a single 5 μg in 20 μl dose without clinical evidence of acute or subacute neurotoxicity. This is equivalent to 250 μg/ml, nearly 6-fold higher than the ID₅₀ doses for these tumors determined by in vitro assays. Although we did not look for histopathological evidence of neurotoxicity, experimental studies of neurotoxicity caused by local central nervous system therapy indicate that behavioral and clinical abnormalities are closely associated with histopathological abnormalities (42, 43).

In summary, we have found that treatment of s.c. implanted glioblastoma and medulloblastoma xenografts with TGF-α-PE38 results in significant tumor regression and growth delay; that treatment of intracranial xenograft implants with i.p. TGF-α-PE38 significantly increased median survival only for tumors with the highest expression of EGFR; but that treatment of intracranial tumors by a single intratumoral injection significantly increased median survival in all
potentially enhancing the cytotoxic effects of this recombinant toxin: by up-regulating EGF receptor in target tumors; or by continuous administration of the recombinant toxin directly into tumor. Indeed, Adachi et al. (44) reported that recombinant tumor necrosis factor-α increases EGFR expression in glialoma cells in vitro and enhances the cytotoxicity of 123I-labeled mAb 425. Repetitive intratumoral drug doses can be administered at the time of surgery or after the implantation of an intratumoral catheter and drug can be administered as an 154. Steiner, B., Leidner, W., and Sabel, M. Improved survival with the use of adjuvant chemotherapy in the treatment of medulloblastoma. J. Neurosurg., 74: 433–440, 1991.


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