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 leptomeningal 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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3 The abbreviations used are: TGF-α, transforming growth factor-α; EGFR, epidermal growth factor receptor; mAb, monoclonal antibody; PE, *Pseudomonas* exotoxin; i.e., intracranial; EGF, epidermal growth factor; ID<sub>50</sub>, drug concentration that results in a 10% survival of colony forming units.

TGF-α<sup>3</sup>-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 M<sub>f</sub> 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 EGF (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
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. 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.

Yeast Ribosomal RNA. Toxin expression was determined by RNA dot blot analyses. 

Xenograft Treatment. Xenograft tumors for intracranial implantation were measured every 2-3 days with vernier calipers (Mano-Cell Corp., New York, NY). Tumor volume was calculated by the following formula (18):

\[ \text{volume} = \frac{\text{width} \times \text{length}}{2} \]

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 cytosieve screen. Dissociated cells were washed through the cytosieve 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 was 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. Omotic 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 (17) 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 the 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 0.9% 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 Frederick, 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 initial 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 the 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 (Mano-Cell Corp., New York, NY). Tumor volume was calculated by the following formula (18):
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 JHG-10 gliomas.

To evaluate the role of EGF 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. 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.

**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. The 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 EGFR than all brain tumor cell lines.

**In Vitro Cytotoxicity.** The results of clonogenic assays of adherent brain tumor cell lines and A431 carcinoma controls 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 ID50 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 ID50 concentrations less than 0.3 ng/ml. Although there was no significant difference in ID50 or drug concentration that results in a 50% survival of colony forming units doses for

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I. Pastan, unpublished observations.

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**Table 1 In vitro sensitivity of human brain tumor cell lines to TGF-α-Pseudomonas exotoxin fusion proteins**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor histology</th>
<th>PE-38 (ng/ml)</th>
<th>TP-40 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U373</td>
<td>Glioblastoma</td>
<td>0.03</td>
<td>0.08</td>
</tr>
<tr>
<td>U251</td>
<td>Glioblastoma</td>
<td>0.11</td>
<td>0.11</td>
</tr>
<tr>
<td>DAOY</td>
<td>Medulloblastoma</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>JHG-31</td>
<td>Glioblastoma</td>
<td>0.28</td>
<td>0.74</td>
</tr>
<tr>
<td>WF</td>
<td>Glioblastoma</td>
<td>0.07</td>
<td>0.33</td>
</tr>
<tr>
<td>U87</td>
<td>Glioblastoma</td>
<td>0.54</td>
<td>1.60</td>
</tr>
<tr>
<td>JHG-10</td>
<td>Pediatric glioblastoma</td>
<td>0.67</td>
<td>2.00</td>
</tr>
<tr>
<td>A431</td>
<td>Epidermoid carcinoma</td>
<td>0.002</td>
<td>0.006</td>
</tr>
</tbody>
</table>

a ID50, drug concentration that results in a 50% survival of colony forming units.

b Cell line derived from a 7-year-old patient.

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**Fig. 2.** Clonogenic assay survival curves for A431 carcinoma and DAOY medulloblastoma treated by continuous exposure to TGF-α-PE38 or TP40. A431 cells, which have the highest EGFR expression, are most sensitive to PE38. DAOY cells treated with TP-40 show a higher percent survival than A431 or DAOY cells treated with PE38. Pretreatment of DAOY cells with 100-fold excess EGF abolishes the cytotoxic effect of PE38 and has a small growth stimulatory effect.
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

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**Table 2** Response of s.c. human brain tumor xenografts to i.p. PE-38

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Treated-control (days)$^a$</th>
<th>$P$ value</th>
<th>Treated/control (% increase mean vol.)$^a$</th>
<th>$P$ value</th>
<th>Tumor regression (no.)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>U373 glioblastoma</td>
<td>27</td>
<td>&lt;0.001</td>
<td>23.2</td>
<td>&lt;0.001</td>
<td>6/10</td>
</tr>
<tr>
<td>U251 glioblastoma</td>
<td>19</td>
<td>&lt;0.001</td>
<td>9.3</td>
<td>&lt;0.001</td>
<td>7/9</td>
</tr>
<tr>
<td>DAOY medulloblastoma</td>
<td>15</td>
<td>&lt;0.001</td>
<td>14.4</td>
<td>&lt;0.001</td>
<td>4/9</td>
</tr>
</tbody>
</table>

$^a$ 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.
responses to conventional chemotherapy in childhood medulloblas-
toma are common, the majority of treatment failures occur in the
primary tumor site, indicating that local control remains a significant
problem. For these reasons, targeted therapy based on selective rec-
ognition of cell-surface antigens or receptors represents an important
alternative approach to conventional chemotherapeutic agents.

Monoclonal antibodies have been raised against various glioma or
neuroectodermal tumor antigens for use in targeted therapy (20–23).
The effectiveness of these therapies, however, may be limited by
tumor heterogeneity, variable expression of tumor-specific antigens,
insufficient cytotoxicity, inefficient internalization processes for anti-
gen-antibody complexes, or inadequate delivery of antibody macro-
molecules across restrictive blood-brain barrier (24–26).

**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. Amplifi-
cation of the EGFR gene has been identified in 17–50% of glioblas-
tomas 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 menin-
gioma (8, 28, 31). By contrast, EGF receptors are few or undetectable
in normal human glial cells and neurons (8, 32), suggesting that
EGFR-targeted local brain tumor therapy should enjoy a relatively
high therapeutic index.

Preclinical and clinical studies of anti-EGFR monoclonal antibod-
ies provide a strong rationale for using targeted therapy for mali-
nant gliomas. Murine IgG2a mAb 425 binds to the EGFR without
stimulating tyrosine kinase activity or other signal transduction path-
ways. 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, ge-
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* ex-
pression 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 EGFR targeted therapy (6, 39, 40). The steps involved in TGF-α-
PE-mediated cytotoxicity include: (a) binding of the TGF-α-toxin com-
plex to EGFR; (b) translocation of the entire complex to the cytosol
in clathrin-coated vesicles; (c) cleavage of the toxin into the translo-
cation 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 *in vitro* and in *in vivo* studies demon-
strated significant cytotoxic activity of TGF-α-PE against human epi-
dermoid 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 cyto-
toxic activity of TGF-α-PE is directly related to EGFR receptor expres-
sion, and that saturation of these receptors by excess EGF completely

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**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.
eliminated the cytotoxic effect of the toxin. Furthermore, PE38 was more active than TP-40 against all brain tumor cell lines, an observation that may be explained by the greater stability of PE38. Accordingly, PE38 was chosen for subsequent studies in vivo in which 3 brain tumor cell lines with a range of expression of EGFR were examined for their sensitivity to TGF-α-PE38, and compared with the sensitivity of A431 epidermoid carcinoma xenografts which express the highest EGFR density and have been previously studied under similar conditions. The experimental conditions for nude mice bearing flank xenografts treated with continuous i.p. TGF-α-PE38 were similar to those of Pai et al. (15) except that the 10% lethal dose for TGF-α-PE38 is higher than for TGF-α-PE40 (75 versus 65 μg/kg/24 h, respectively).

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 ID<sub>50</sub> 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
xenografts studied. These results suggest several means for 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 glioma cells in vitro and enhances the cytotoxicity of 12SI-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 intermittent or continuous infusion (45). Recently, we have developed a polymeric drug delivery system that permits prolonged drug delivery directly into the brain of patients undergoing surgery. The pharmacology, neurotoxicity, and therapeutic efficacy of this approach have been evaluated in preclinical studies (46, 47), and clinical trials with controlled release polymers impregnated with carmustine are currently under way (48). The demonstrated activity of TGF-α-P38 in human brain tumor xenograft models, together with the frequency of EGFR overexpression in malignant gliomas and other brain tumors, provide a strong rationale for further development of TGF-α-P38 recombinant toxins in brain tumor therapy.

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REFERENCES


Table 3 Response of i.c. xenograft tumor implants in athymic mice to i.p. or intratumoral PE-38

<table>
<thead>
<tr>
<th>Tumor</th>
<th>iP 431</th>
<th>Control</th>
<th>% IMS 431</th>
<th>iP 447</th>
<th>Control</th>
<th>% IMS 447</th>
<th>iP 425</th>
<th>Control</th>
<th>% IMS 425</th>
<th>iP 427</th>
<th>Control</th>
<th>% IMS 427</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-251 glioblastoma</td>
<td>50(15-62f)</td>
<td>41(34-49)</td>
<td>24.4</td>
<td>36(24-47)</td>
<td>56(18-78)</td>
<td>0.05</td>
<td>33(14-37)</td>
<td>55.5</td>
<td>0.049</td>
<td></td>
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<tr>
<td>DAOY medulloblastoma</td>
<td>44(18-58)</td>
<td>38(29-47)</td>
<td>15.7</td>
<td>22(17-33)</td>
<td>42(18-58)</td>
<td>0.035</td>
<td>23(15-30)</td>
<td>73.9</td>
<td>0.023</td>
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</tr>
<tr>
<td>A431 epidermoid carcinoma</td>
<td>34(23-48)</td>
<td>22(17-33)</td>
<td>54.5</td>
<td>22(17-33)</td>
<td>42(18-58)</td>
<td>0.0037</td>
<td>23(15-30)</td>
<td>73.9</td>
<td>0.0013</td>
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</tr>
</tbody>
</table>

f Single intratumoral injection 14 days after intracranial tumor implantation.

* Range of survival expressed in days.

f +, one PE38-treated animal that survived longer than 98 days.


Transforming Growth Factor-\(\alpha\)---\textit{Pseudomonas} Exotoxin Fusion Protein (TGF-\(\alpha\)-PE38) Treatment of Subcutaneous and Intracranial Human Glioma and Medulloblastoma Xenografts in Athymic Mice

Peter C. Phillips, Cindee Levow, Mark Catterall, et al.


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