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Transforming growth factor α–*Pseudomonas* exotoxin fusion protein prolongs survival of nude mice bearing tumor xenografts

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ABSTRACT **Transforming growth factor α (TGFα)–*Pseudomonas* exotoxin 40 (PE40) is a chimeric protein consisting of an N-terminal TGFα domain fused to a C-terminal 40-kDa segment of the *Pseudomonas* exotoxin A protein. TGFα–PE40 exhibits the receptor-binding activity of TGFα and the cell-killing activity of PE40. These properties make TGFα–PE40 an effective cytotoxic agent for cells that possess epidermal growth factor receptors (EGFR). However, the utility of this protein as an anticancer agent has been unclear because many normal tissues express EGFR and may be damaged by exposure to TGFα–PE40. To address this issue, we injected nude mice with a lethal inoculum of either A431 or HT29 human tumor cells that possess EGFR or with Chinese hamster ovary (CHO) tumor cells that lack EGFR. Animals were treated with a derivative of TGFα–PE40 in which the cysteine residues are replaced by alanine, termed "TGFα–PE40δcys," or with saline once a day for 5 days. Mice bearing EGFR$^+$ tumor cells lived significantly ($P < 0.001$) longer when treated with TGFα–PE40δcys compared with saline-treated controls (median survival: A431 cells, 51.5 vs. 25.5 days; HT29 cells, 101 vs. 47.5 days). TGFα–PE40δcys did not prolong the survival of mice bearing tumor cells that lack EGFR (median survival: CHO cells, 15.5 vs. 19.5 days). The only toxicity to normal tissues was mild periportal hepatic necrosis. These studies indicate that a therapeutic window exists *in vivo* for the use of some growth factor-toxin fusion proteins as anticancer agents.**

TGFα–PE40 [transforming growth factor α (TGFα)–*Pseudomonas* exotoxin 40-kDa fragment] is a chimeric protein composed of two independent domains that are produced as a single recombinant fusion protein in bacteria (1–4). The TGFα domain is derived from a synthetic gene encoding the mature form of human TGFα (5). The PE40 domain is derived from the *Pseudomonas* exotoxin A gene of *Pseudomonas aeruginosa* (6). TGFα–PE40 is a bifunctional molecule that possesses both "cell-targeting" and cell-killing activities. TGFα functions as the cell-targeting domain of TGFα–PE40 by binding specifically to the surface of cells that possess epidermal growth factor receptors (EGFR) (1, 2). PE40 functions as the cell-killing domain. PE40 kills mammalian cells by ADP-ribosylating elongation factor 2, which results in the inhibition of protein synthesis (7). This combination of cell killing and cell-targeting properties suggests that TGFα–PE40 may have utility as a selective cytotoxic agent.

Many human tumor cells possess EGFR, and some tumor types exhibit increased numbers of EGFR relative to normal tissues (8–12). The association of EGFR with human cancer cells makes this receptor an attractive target for the selective delivery of anticancer agents. One method of exploiting EGFR overexpression on human tumor cells is to construct hybrid molecules consisting of potent cell poisons linked to growth factors that bind to EGFR. This concept was first developed by conjugating epidermal growth factor to *Pseudomonas* exotoxin and using this compound to kill cells that possess EGFR (13, 14). This approach was later refined by defining a 40-kDa segment of *Pseudomonas* exotoxin, PE40, that is missing the binding domain but retains the translocating and ADP-ribosylating activity of native *Pseudomonas* exotoxin (15–17). A series of hybrid proteins were created with this truncated form of *Pseudomonas* exotoxin fused to TGFα and other ligands (2, 18–20).

The TGFα–PE40 hybrid proteins kill mammalian cells that possess EGFR but are relatively harmless to cells that lack EGFR. *In vitro*, TGFα–PE40 is a potent cell poison that has been shown to intoxicate cells via specific binding to EGFR (1, 2). However, it has been unclear whether TGFα–PE40 would prove useful as an antitumor agent *in vivo* because many normal tissues possess EGFR. These tissues might be severely damaged by exposure to TGFα–PE40, thereby limiting the amount of drug that could be safely administered to cancer patients. The current studies were undertaken to assess the relative therapeutic-to-toxic effect of TGFα–PE40 and to evaluate the overall effectiveness of TGFα–PE40 as an antitumor agent in animals.

MATERIALS AND METHODS

Cell Lines. A431 epithelial carcinoma cells, HeLa cells, and Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection and used here for cell-killing assays (21). A431 cells were also used to prepare plasma membrane vesicles for EGFR binding assays (22). All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum and antibiotics.

Cell-Killing Assays. The cell-killing assays were performed with the cell lines indicated in the text. Each cell line was seeded into 96-well plates at 10,000 viable cells per well. Twenty-four hours later, the cells were washed once and placed in serum-free medium containing the test compound under study. Forty-eight hours later, the number of surviving cells was quantitated by using a 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described (23).

Animal Studies. Six- to 8-week-old female nude mice were purchased from Harlan–Sprague–Dawley. Animals were housed in laminar flow biosafety hoods and fed sterilized lab chow. Tumor cell injections were performed with 27-gauge

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Abbreviations: TGFα, transforming growth factor α; PE40, *Pseudomonas* exotoxin 40-kDa fragment; EGFR, epidermal growth factor receptor(s).

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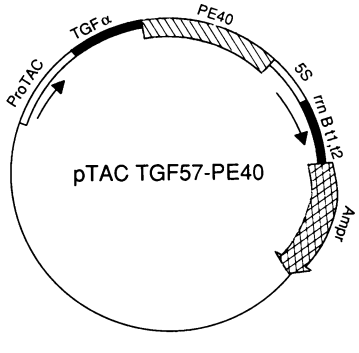
needles. All animals received 20 million tumor cells in 1 ml of phosphate-buffered saline (PBS) by midabdominal injections. Twenty million tumor cells represented an inoculum at least 10 times the minimal lethal dose for each cell line. All treatments consisted of once-a-day injections of test proteins or PBS for 5 days.

Recombinant DNA plasmids were grown, and fusion proteins were produced in *Escherichia coli* strain JM109. TGFα–PE40δcys protein, in which the four cysteine residues of PE40 are replaced by alanine, was produced and purified as described (2). All protein analytical measurements including gel electrophoresis, immunoblotting (Western blotting), N-terminal sequencing, and ADP-ribosylating activity were also measured as described (2). EGFR binding inhibition assays were performed with A431 cell membrane vesicles (24).

Iodoacetic Acid Treatment. TGFα–PE40δcys (22 μM) was denatured in 6 M urea/1 mM EDTA/50 mM Hepes, pH 8.0. Reduction of disulfide bonds was effected by the addition of dithiothreitol to a final concentration of 2.2 mM and incubation at room temperature for 4 hr. A freshly prepared solution of sodium iodoacetate was then added to a final concentration of 4.4 mM, and the incubation was continued in the dark for 90 min. The reaction mixture was then extensively dialyzed against PBS at 4°C.

RESULTS

Production of Fusion Protein. To produce sufficient quantities of TGFα–PE40 for use in animal experiments, we constructed the plasmid vector pTAC-TGF57-PE40 shown in Fig. 1. This bacterial expression system was adopted from the work of Linemeyer *et al.* (25), who optimized this vector for expression of mammalian cell proteins. pTAC-TGF57-PE40 expresses a synthetic gene encoding mature human TGFα fused to a PE40 gene that contains alanine codons in place of the four cysteine codons normally present in the PE gene (2). The fusion protein encoded by this genetic construction was designated TGFα–PE40δcys. TGFα–PE40 and TGFα–PE40δcys both specifically bind to and kill EGFR-bearing cells *in vitro*, although when compared in the same assay, TGFα–PE40 is ≈10 times more potent at cell killing than TGFα–PE40δcys (ref. 2 and unpublished data). However, TGFα–PE40δcys is easier to isolate in large quantities. Therefore, TGFα–PE40δcys was used in place of TGFα–PE40 for our *in vivo* studies. The pTAC-TGF57-PE40 plasmid was introduced into JM109 *E. coli*, the bacteria were grown to high density, and the plasmid was induced with isopropyl β-D-thiogalactopyranoside. TGFα–PE40δcys was purified from these cultures through multiple chromatographic steps, including size-exclusion and ion-exchange chromatography (Fig. 1). The final purity (>95%) of the protein product was established by SDS/PAGE, Western blotting with both anti-TGFα and anti-PE40 antisera, N-terminal protein sequencing, and amino acid composition analysis (2). Preservation of the enzymatic activity of the toxin domain of TGFα–PE40δcys was confirmed by measuring the ADP-ribosylating activity (26, 27) of the fully purified protein (specific activity, 20.0 nmol of ADP-ribose per mg/min).



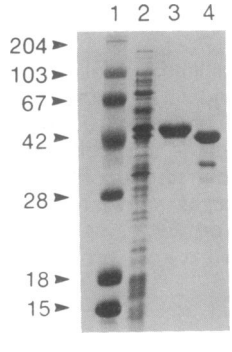


FIG. 1. Recombinant expression of TGFα–PE40δcys. (*Upper*) Schematic representation of recombinant plasmid expression vector used to produce TGFα–PE40δcys in *E. coli*. (*Lower*) Polyacrylamide gel electrophoresis analysis of TGFα–PE40δcys and PE40. Lanes: 1, molecular weight markers (shown × 10^{-3}); 2, crude lysate of *E. coli* producing TGFα–PE40δcys; 3, purified TGFα–PE40δcys; and 4, purified PE40.

Antiproliferative Activity *in Vitro*. The spectrum of tumor cell types that are sensitive to the cell-killing activity of TGFα–PE40δcys was examined by using a series of normal and transformed cell lines. These cell lines were exposed to TGFα–PE40δcys, native PE toxin, or PE40 without an associated growth factor domain and were analyzed for cell viability 48 hr later (23). Table 1 lists the concentrations of TGFα–PE40δcys needed to reduce the viable cell populations by 50% (ED_{50}). All $EGFR^+$ cell lines exhibited ED_{50} values between 70 pM and 200 nM. As expected, cell lines that did not express EGFR (e.g., CHO) were highly resistant to TGFα–PE40δcys. The failure of TGFα–PE40δcys to kill $EGFR^-$ cell lines cannot be attributed to an intrinsic resistance of these cells to the mechanism of intoxication used by PE toxin because both $EGFR^+$ and $EGFR^-$ cell lines were efficiently killed by native PE toxin (Table 1).

Several lines of evidence suggest that the cell-killing activity of TGFα–PE40δcys is mediated by specific binding to EGFR. Purified recombinant PE40 was much less cytotoxic (median, 297-fold; range, 32- to 2776-fold) than TGFα–PE40δcys when tested on the same cell lines. Similarly, when the specific receptor binding activity of TGFα–PE40δcys was disrupted by eliminating the disulfide bonds within the TGFα domain, the resulting protein was much less efficient at killing tumor cells. The disulfide bonds within TGFα–PE40δcys were irreversibly eliminated by treatment with dithiothreitol

Table 1. Cell killing by TGFα–PE40δcys, native *Pseudomonas* (PE) toxin, and PE40 *in vitro*

Cell lines	EGFR × 10^{-4} per cell	EC_{50} cell killing, pM: TGFα–PE40δcys	PE toxin	PE40
$EGFR^+$				
A431	250	110	20	31,000
BT-20	12	90	220	35,000
HT29	4.4	670	1200	>300,000
HeLa	33	3900	1700	124,000
SCC-4	17	450	150	43,000
SCC-25	10	70	60	186,000
MDA-MB-468	160	250	2000	68,000
U373MG	1.7	200,000	80	NT
$EGFR^-$				
CHO	—	>300,000	960	>300,000
NR-6	—	>300,000	50	NT

NT, not tested.

and iodoacetic acid, CH_2ICOOH. The CH_2ICOOH-treated TGFα–PE40δcys did not exhibit EGFR-binding activity (IC_{50} receptor binding inhibition: TGFα–PE40δcys, 22 nM; CH_2-ICOOH-treated TGFα–PE40δcys, >1.0 μM) but retained full enzymatic ADP-ribosylating activity. The CH_2ICOOH-treated TGFα–PE40δcys was less potent by a factor of 285 than untreated TGFα–PE40δcys as a cytotoxic agent for A431 cells (ED_{50} cell killing, 31,300 vs. 100 pM).

Antitumor Activity *in Vivo*. To evaluate the antitumor activity of TGFα–PE40δcys *in vivo*, one highly sensitive (A431) and one moderately sensitive (HT29) human tumor cell line were selected for transplantation into nude mice. Preliminary studies indicated that 2×10^7 A431 or HT29 cells injected i.p. into nude mice produced a uniformly lethal tumor burden within 1–2 months. Dose-ranging experiments indicated that a single i.p. injection of 75 μg of TGFα–PE40δcys was lethal to 50% of the animals (LD_{50}). However, no animals died following multiple (up to five) daily exposures to TGFα–PE40δcys at 25 μg per injection. Separate groups of mice were inoculated with either 2×10^7 A431 or HT29 cells. Beginning 24 hr later, each animal received daily i.p. injections of PBS or 25 μg of TGFα–PE40δcys in PBS for the next 5 days. The median survival time for mice bearing A431 tumors was 25.5 days for mice treated with PBS and 51.5 days for mice receiving TGFα–PE40δcys (Fig. 2 *Upper*). This difference was highly statistically significant ($P < 0.001$) in a logarithmic rank survival analysis (28). Mice inoculated with HT29 cells exhibited median survivals of 47.5 days when treated with PBS and 101 days after receiving TGFα–PE40δcys ($P < 0.001$) (Fig. 2 *Lower*). The efficacy of TGFα–PE40δcys was also assessed in animals injected with A431 cells and allowed to incubate their tumors for 1 week prior to the initiation of therapy. These mice received daily injections of PBS or TGFα–PE40δcys (25 μg) on days 8 through 12 after tumor cell inoculation. The median survival of animals receiving PBS was 32.5 days vs. 44.5 days for animals treated with TGFα–PE40δcys ($P < 0.004$).

No evidence of treatment-associated toxicity (e.g., early deaths or weight loss) was noted in any of the groups receiving TGFα–PE40δcys. All animals had maintained or

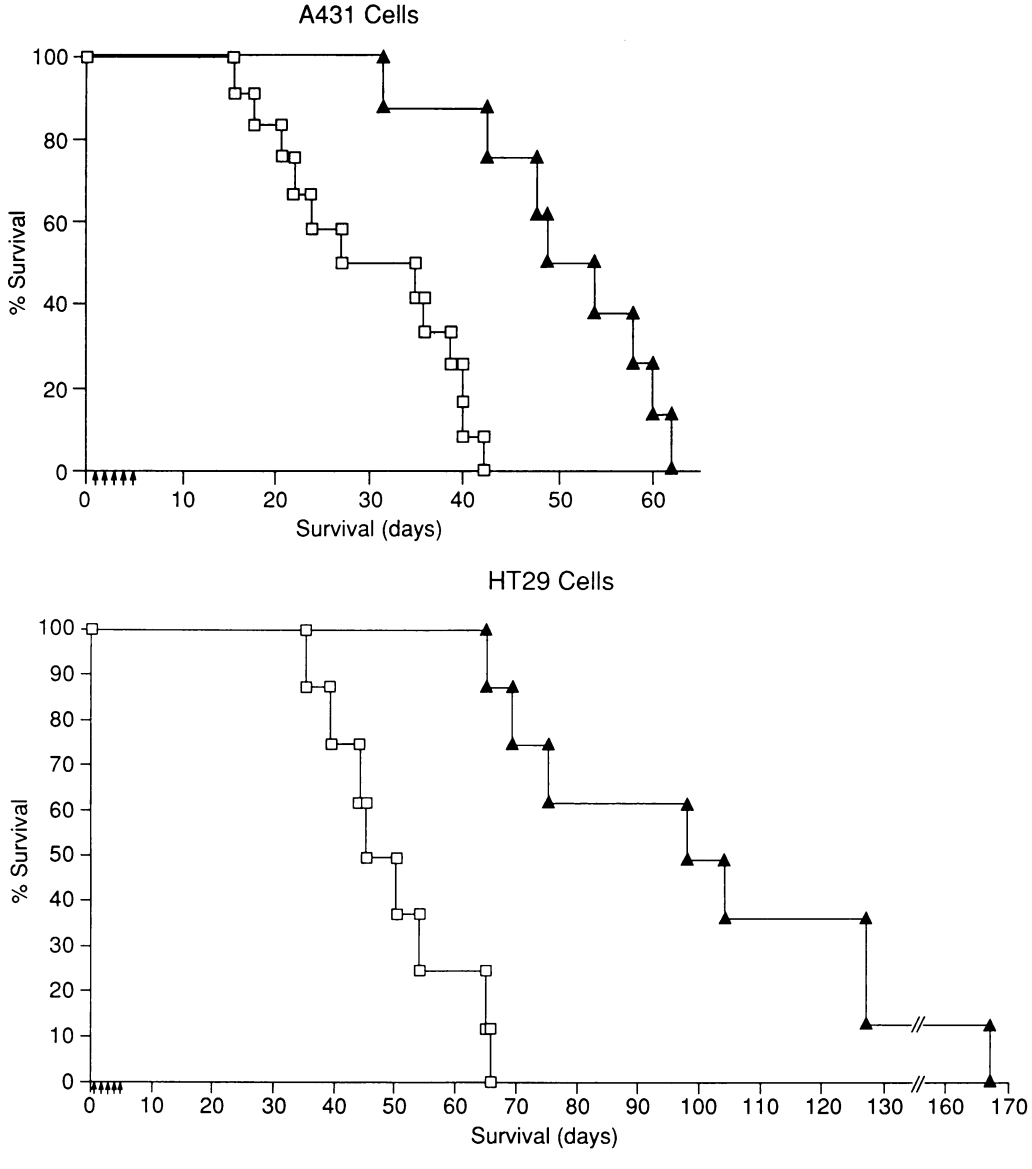


FIG. 2. Survival of nude mice bearing human tumor xenografts. (*Upper*) Survival curves for nude mice inoculated i.p. with 2×10^7 A431 tumor cells. □, Survival of mice treated with PBS daily for 5 days ($n = 12$); ▲, survival of mice treated with TGFα–PE40δcys daily for 5 days ($n = 8$). Arrows indicate treatment days. (*Lower*) Survival curves for nude mice inoculated i.p. with 2×10^7 HT29 tumor cells. □, Survival of mice treated with PBS daily for 5 days ($n = 8$); ▲, survival of mice treated with TGFα–PE40δcys daily for 5 days ($n = 8$). Arrows indicate treatment days.

marginally increased their pretreatment body weights by 2 weeks after tumor inoculation [starting weights: range, 19.8–26.6 g (mean, 22.9 g); weights after 14 days: range, 21.4–27.1 g (mean, 24.3 g)]. A parallel group of mice treated with TGFα–PE40δcys (25 μg per day for 5 days) but not receiving tumor cells was sacrificed and subjected to full autopsy and histologic examination. The only evidence of tissue damage in these animals consisted of slight hepatomegaly and mild periportal (zone I) necrosis. Tissue samples from brain, heart, lung, spleen, kidneys, gastrointestinal tract, and bone marrow were unremarkable. Animals exposed to a higher dose of TGFα–PE40δcys (75 μg) as a single i.p. injection exhibited more extensive hepatic damage but showed no evidence of toxicity to other tissues.

Two types of control experiments were carried out to evaluate the specificity of TGFα–PE40δcys therapy *in vivo*. First, nude mice were injected with a lethal inoculum of EGFR-deficient CHO cells and treated with TGFα–PE40δcys or saline. There was not statistically significant difference in the survival times between these groups of mice (median survival: TGFα–PE40δcys-treated, 15.5 days; saline-treated, 19.5 days). Second, nude mice were injected with a lethal inoculum of A431 cells and treated with TGFα–PE40δcys, saline, or CH_2ICOOH-treated TGFα–PE40δcys. Again, TGFα–PE40δcys treatment prolonged the survival of the $EGFR^+$ tumor-bearing mice (Fig. 3). However, tumor-bearing animals treated with CH_2ICOOH-treated TGFα–PE40δcys did not live longer than animals receiving saline (median survival time: 49 and 53 days, respectively).

DISCUSSION

Our studies indicate that a broad range of histologic cell types are susceptible to the cell-killing activity of TGFα–PE40δcys, including cancer cells derived from breast, brain, cervix, colon, and oral squamous epithelial tumors (Table 1). Additional studies with tumor cells from lung, ovary, endometrium, and stomach cancers indicate that TGFα–PE40δcys is also an effective antiproliferative agent for these tumor cells (data not shown). The common attribute among these cell lines is the expression of EGFR on their cell surface. $EGFR^-$ cells (e.g., CHO or NR-6) are highly resistant to the cytotoxic activity of TGFα–PE40δcys. The degree of susceptibility of different tumor cell lines to the cell-killing activity of TGFα–PE40δcys may be related in part to the number of EGFR present on each cell line. For example, A431 cells (29–31) possess $2–3 \times 10^6$ EGFR per cell and exhibited an ED_{50} of 110 pM. HT29 cells (32) possess 4.4×10^4 EGFR per cell and required 6.0 times more TGFα–PE40δcys to produce an equivalent level of cell killing (ED_{50}, 669 pM). However, EGFR numbers are not the only factor governing susceptibility to TGFα–PE40δcys since other tumor cell lines with relatively few EGFR compared with A431 cells (e.g., BT-20, 1.2×10^5 EGFR per cell) were also readily killed by TGFα–PE40δcys (ED_{50}, 94 pM).

The requirement of EGFR on tumor cells for TGFα–PE40δcys to produce an antitumor effect *in vivo* is suggested by the observation that mice bearing CHO cell tumors are not benefited by TGFα–PE40δcys therapy. A more rigorous demonstration of the critical involvement of EGFR binding in the mechanism of TGFα–PE40δcys-induced cell killing is provided by an analysis of CH_2ICOOH-treated TGFα–PE40δcys. The only disulfide bonds in the TGFα–PE40δcys species used in our studies are located in the TGFα domain (2). Therefore, treatment with dithiothreitol and CH_2ICOOH should only affect TGFα–PE40δcys properties that depend on the disulfide-constrained conformation of TGFα. As expected, the CH_2ICOOH-treated TGFα–PE40δcys species exhibited the same enzymatic activity as unmodified TGFα–PE40δcys but was not able to specifically bind EGFR. Concomitant with this loss in receptor binding activity, the CH_2ICOOH-treated TGFα–PE40δcys exhibited dramatically reduced cell-killing activity in cell culture and failed to prolong the survival of tumor-bearing mice. Similarly, recombinant PE40 protein that lacks a TGFα domain was much less effective than TGFα–PE40δcys at killing $EGFR^+$ tumor cells *in vitro* (Table 1). This result is consistent with earlier experiments by Lorberboum-Galski *et al.* (33), which showed

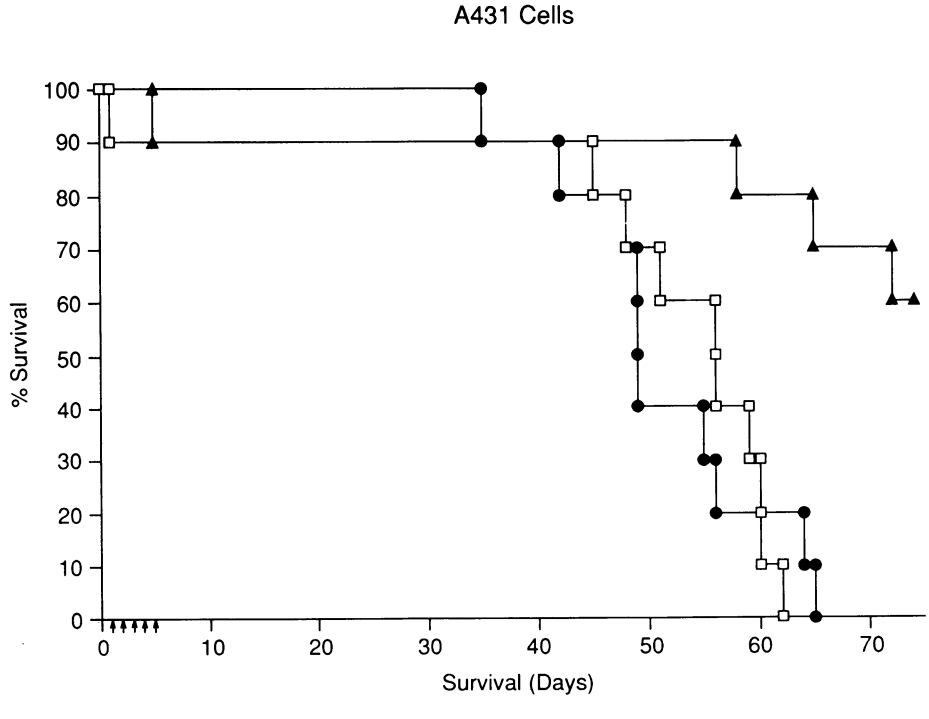


FIG. 3. Survival of nude mice bearing A431 cell xenografts. Survival curves for nude mice inoculated i.p. with 2×10^7 A431 tumor cells. □, Survival of mice treated with PBS (n = 10); ▲, survival of mice treated with TGFα–PE40δcys (n = 10); ●, survival of mice treated with iodoacetic acid-treated TGFα–PE40δcys (n = 10). Arrows indicate treatment days.

that purified PE40 was ineffective at killing target cells *in vivo*. Each of these studies suggests that a biologically active TGFα domain capable of binding to cellular EGFR is required for TGFα–PE40δcys to effectively kill tumor cells that express EGFR. Conversely, tumor cells must possess biologically available EGFR for TGFα–PE40δcys to be useful as an antiproliferative agent for these cells (1, 2). While the binding of TGFα–PE40δcys to EGFR on tumor cells is clearly necessary for efficient cell killing, receptor occupation itself is unlikely to inhibit tumor growth. For example, HT29 cell proliferation is not suppressed by exposure to TGFα *in vitro* at concentrations up to 1.0 μM. Taken together, these studies indicate that the antitumor effect of TGFα–PE40δcys results from the unique combination of receptor binding and cell intoxication properties exhibited by this fusion protein.

The current study shows that TGFα–PE40δcys therapy significantly prolongs the survival of mice bearing human tumor cell xenografts. The survival benefit was greatest when therapy was started 24 hr after the tumor cells were inoculated. However, even after delaying therapy for 1 week, the animals receiving TGFα–PE40δcys lived significantly longer than control animals treated with saline. These observations suggest that hybrid growth factor–toxin molecules directed against tumor cells that express EGFR can be effective anticancer agents. Surprisingly, TGFα–PE40δcys caused only minimal damage to normal tissues despite the presence of EGFR on many normal cell types (34–36). A possible explanation for the lack of toxicity associated with TGFα–PE40δcys therapy is that normal nondividing cells may be less susceptible to the cytotoxic effect of protein synthesis inhibition than rapidly dividing tumor cells. Alternatively, many normal cell populations may be protected from TGFα–PE40δcys by exhibiting fewer numbers of EGFR than tumor cells. Further studies are needed to clarify this point and establish why TGFα–PE40δcys-induced cell killing is selective for tumor cells *in vivo*. However, the current observations represent a significant change compared with traditional anticancer chemotherapeutics that exhibit narrow safety margins *in vivo*. Early clinical trials should determine if the observed safety and efficacy of TGFα–PE40δcys in mice can be reproduced in man.

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