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Biological activity of a transforming growth factor-alpha – *Pseudomonas* exotoxin fusion protein in vitro and in vivo

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SUMMARY

Transforming growth factor-alpha (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. In the current study, we report that a modified TGFα-PE40 derivative significantly prolongs the survival of nude mice bearing tumors derived from cell lines which express the epidermal growth factor receptor (EGFR). In addition, the therapeutic benefit of this protein is mediated by specific binding to the EGF receptor. These results indicate that a therapeutic window exists in vivo for the use of some growth factor – toxin fusion proteins as anticancer agents.

INTRODUCTION

Many human tumor cells possess epidermal growth factor receptors (EGFR) and some tumor types exhibit increased numbers of EGFR relative to normal tissues [6,7]. The association of EGFR with human cancer cells makes this receptor an attractive target for the selective delivery of anticancer agents.

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 EGFR [1,2]. PE40, which is the C-terminal 40-kDa fragment of pseudomonas exotoxin A, functions as the cell killing domain. PE40 kills mammalian cells by ADP-ribosylating elongation factor 2, which results in the inhibition of protein synthesis [3,4]. This combination of cell killing and cell targeting properties suggests that TGFα-PE40 may have utility as a selective cytotoxic agent for cells expressing EGFR.

The current studies were undertaken to assess the relative in vivo therapeutic effect of TGFα-PE40 and to evaluate the overall effectiveness of TGFα-PE40 as an antitumor agent in animals.

MATERIALS AND METHODS

Biochemistry.

The plasmid vector 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-PE40δcys protein was produced in *E. coli* and purified as previously described [2].

Iodoacetic acid-treated TGFα-PE40δcys was prepared by denaturing TGFα-PE40δcys in 6 M urea, 1 mM EDTA, and 50 mM HEPES at pH 8.0. Reduction of disulfide bonds was effected by the addition of dithiothreitol (DTT) to a final concentration of 2.2 mM and incubation at room temperature for 4 h. A freshly prepared solution of sodium iodoacetate was then added to a final concentration of 4.4 mM and the incubation continued in the dark for 90 min. The reaction mixture was then extensively dialyzed against PBS at 4 °C.

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Biology

Cell kill and EGFR binding assays were performed as previously described [2]. Briefly, cell kill assays were performed by incubating cell lines plated in 96 well plates with the protein indicated for 48 h, at which time the cells were stained with [3,(4,5-dimethyl-thiazoyl-2-yl)2,5-diphenyltetrazolium bromide]. EGFR binding assays were performed by plating the cells in 24-well plates and incubating with ^{125}I-EGF. With the exception of the NR-6 cell line [5], all cell lines tested are available from the American Type Culture Collection. Animal studies were performed in 6–8-week-old female nude mice purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). All animals received $20 \cdot 10^6$ tumor cells in 1 ml of phosphate buffered saline (PBS) by mid-abdominal injections. Twenty-million tumor cells represented an inoculum at least 10 × the minimal lethal dose for each cell line. All treatments consisted of once a day injections of test proteins or PBS for 5 days.

RESULTS

TGFα-PE40δcys both specifically bind to and kill EGFR-bearing cells in vitro although when compared in the same assay TGFα-PE40 is approximately 10 × more potent at cell killing than TGFα-PE40δcys (Edwards et al. [2], and unpublished data). However, TGFα-PE40δcys is easier to isolate in large quantities, and binds with higher affinity to the EGF receptor. This higher affinity suggested that the cell killing effect of the δcys derivative might be more specific for cells expressing the EGF receptor. Therefore, TGFα-PE40δcys was used in our in vivo studies.

Antiproliferative activity in vitro

The spectrum of tumor cell types that are sensitive to the cell killing activity of TGFα-PE40δcys, native PE toxin, and PE40 was examined using a series of normal and transformed cell lines. All EGFR-positive cell lines exhibited EC_{50}s for TGF-PE40δcys between 70 pM and 200 nM (Table 1). 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-deficient cell lines cannot be attributed to an intrinsic resistance of these cells to the mechanism of intoxication employed by PE toxin since both EGFR-positive and EGFR-deficient 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 via specific binding to EGFR. Purified recombinant PE40 was much less cytotoxic than TGFα-PE40δcys when tested on the same cell lines. Similarly, if the specific receptor binding activity of TGFα-PE40δcys is disrupted by eliminating the disulfide bonds within the TGFα domain, the resulting protein is much less efficient at killing tumor cells. The disulfide bonds within TGFα-PE40δcys were irreversibly eliminated by treatment with DTT and iodoacetic acid (IAA). The IAA-treated TGFα-PE40δcys did not exhibit EGFR

TABLE 1

Cell killing by TGFα-PE40δcys, PE toxin, and PE40 in vitro

	EGFR/Cell	EC_{50} cell killing (pM)		
		TGFα-PE40δcys	PE toxin	PE40
EGFR-positive cell lines				
A431	2.5×10^6	110	20	31000
MDA-MB-468	1.6×10^6	250	2000	68000
HELA	3.3×10^5	3900	1700	124000
SCC-4	1.7×10^5	450	150	43000
BT-20	1.2×10^5	90	220	35000
SCC-25	1.0×10^5	70	60	186000
HT29	4.4×10^4	670	1200	>300000
U373MG	1.7×10^4	200000	80	NT[a]
EGFR-deficient cell lines				
CHO	–	$>3 \times 10^5$	960	$>3 \times 10^5$
NR-6	–	$>3 \times 10^5$	50	NT

NT = not tested.

binding activity (IC_{50} receptor binding inhibition: TGFα-PE40δcys = 22 nM, IAA-treated TGFα-PE40δcys = > 1.0 μM), but retained full enzymatic ADP-ribosylating activity. The IAA-treated TGFα-PE40δcys was 285-fold less potent than untreated TGFα-PE40δcys as a cytotoxic agent for A431 cells (EC_{50} cell killing = 31300 vs. 110 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. Separate groups of mice were inoculated with 2×10^7 A431 or HT29 cells. Beginning 24 h later, each animal received daily i.p. injections of PBS or 25 μg of TGFα-PE40δcys (1/3 the LD_{50}) 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. 1A). This difference was highly statistically significant ($P < 0.001$). 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. 1B). The efficacy of TGFα-PE40δcys was also assessed in animals injected with A431 cells and allowed to incubate their tumors for one week prior to the initiation of therapy. The median survival of animals receiving PBS was 32.5 days vs. 44.5 days for animals treated with TGFα–PE40δcys ($P < 0.004$).

Two types of control experiments were carried out to

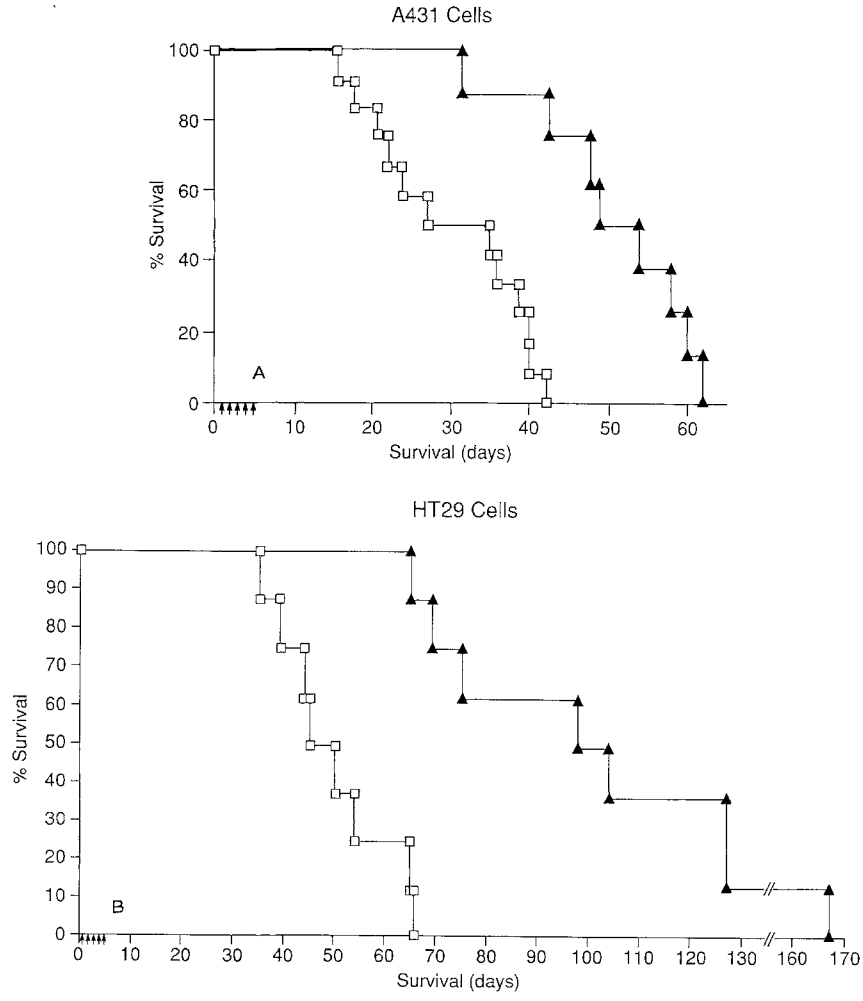

Fig. 1. Survival of nude mice bearing human tumor xenografts. **A.** Survival curves for nude mice inoculated i.p. with 2×10^7 A431 tumor cells. Open squares indicate survival of mice treated with PBS daily for 5 days ($n = 12$). Closed triangles indicate survival of mice treated with TGFα-PE40δcys daily for 5 days ($n = 8$). Arrows indicate treatment days. **B.** Survival curves for nude mice inoculated i.p. with 2×10^7 HT29 tumor cells. Open squares indicate survival of mice treated with PBS daily for 5 days ($n = 8$). Closed triangles indicate survival of mice treated with TGFα-PE40δcys daily for 5 days ($n = 8$). Arrows indicate treatment days.

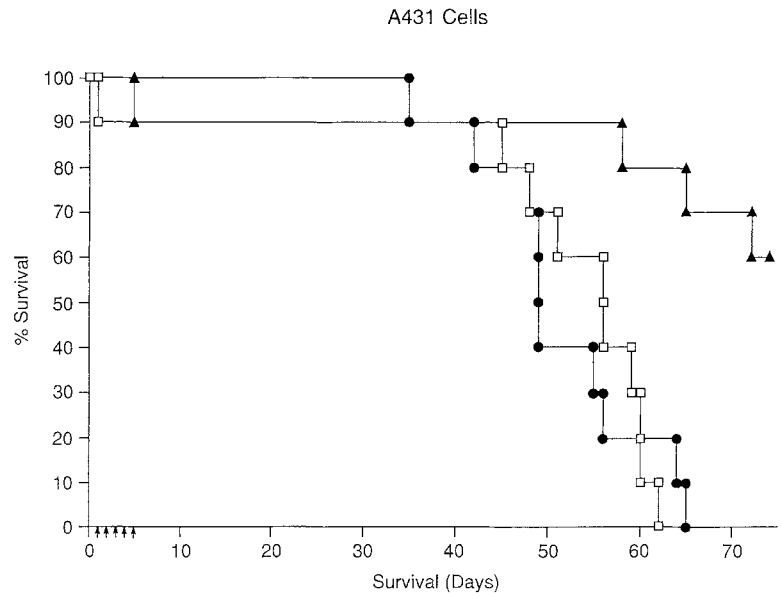

Fig. 2. Survival of nude mice bearing A431 cell xenografts. Survival curves for nude mice inoculated i.p. with 2×10^7 A431 tumor cells. Open squares indicate survival of mice treated with PBS (n = 10). Closed triangles indicate survival of mice treated with TGFα-PE40δcys (n = 10). Closed circles indicate survival of mice treated with iodoacetic acid-treated TGFα-PE40δcys (n = 10). Arrows indicate treatment days.

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 no statistically significant difference in the survival times between these groups of mice (median survival: TGFα-PE40δcys = 15.5 days, saline = 19.5 days). Second, nude mice were injected with a lethal inoculum of A431 cells and treated with either TGFα-PE40δcys, saline, or IAA-treated TGFα-PE40δcys. Again, TGFα-PE40δcys treatment prolonged the survival of the EGFR-positive tumor bearing mice (Fig. 2). However, tumor-bearing animals treated with IAA treated TGFα-PE40δcys did not live longer than animals receiving saline (median survival: 49 and 53 days, respectively).

DISCUSSION

Our studies indicate that a broad range of histologic cell types are susceptible to TGFα-PE40δcys's cell killing activity including cancer cells derived from breast, brain, cervix, colon, and oral squamous epithelial tumors (Table 1). The common attribute among these cell lines is the expression of EGFR on their cell surface. EGFR deficient cells (e.g. CHO or NR-6) are highly resistant to TGFα-PE40δcys's cytotoxic activity. However, EGFR numbers are not the only factor governing susceptibility to TGFα-PE40δcys since other tumor cell lines with relatively few EGFR compared to A431 cells were also readily killed by TGFα-PE40δcys (e.g. BT-20, 1.2×10^5 EGFR per cell: EC_{50} = 90 pM). Other factors which might influence the toxicity of this fusion protein include the rate of new protein synthesis within the cell, variations in endosomal acidification and proteolytic activities, and the sensitivity of each cell line to the effects of ADP-ribosylation of EF-2.

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 benefitted 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 the results obtained with IAA-treated TGFα-PE40δcys. Concomitant with the loss in receptor binding activity, IAA-treated TGFα-PE40δcys exhibited dramatically reduced cell killing activity in cell culture and failed to prolong the survival of tumor-bearing mice. These studies suggest that a biologically active TGFα domain capable of binding to cellular EGFR is required for TGFα-PE40δcys to effectively kill tumor cells in vivo.

The current study demonstrates 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 h after the tumor cells were inoculated. However, even after delaying ther-

apy 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.

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