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TGFα-anti-Tac(Fv)-PE40: A BIFUNCTIONAL TOXIN CYTOTOXIC FOR CELLS WITH EGF OR IL2 RECEPTORS

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Summary Conventional immunotoxins and chimeric toxins made in bacteria are directed to only one receptor or antigen on target cells. In this report we describe the construction of a chimeric molecule TGFα-antiTac(Fv)-PE40 which is composed of human transforming growth factor type α attached to anti-Tac(Fv) which is in turn attached to PE40, a form of *pseudomonas* exotoxin, devoid of its cell recognition domain. TGFα-anti-Tac(Fv)-PE40 is a bifunctional toxin that is produced in *E. coli* and is active on cells bearing either IL2 or EGF receptors.

Introduction Immunotoxins are cytotoxic agents created by attaching protein toxins to tumor-specific monoclonal antibodies (1-3). Immunotoxins produced in this manner have several drawbacks which include low yields, the formation of heterogenous products, and difficulties in large scale production. To circumvent some of these difficulties, we have previously constructed a recombinant single chain antibody toxin fusion protein, anti-Tac(Fv)-PE40, which contains the variable heavy and light chains of the anti-Tac antibody attached to PE40, a recombinant form of pseudomonas exotoxin, which lacks the binding domain of the toxin. This chimeric toxin kills cells bearing the p55 subunit of the IL2 receptor (4). Receptor specific chimeric toxins have also been constructed by attaching CD4 or a variety of growth factors (TGFα, IL2, IL4 , IL6) to PE40 (5-10) or by attaching IL2 to a mutant form of diphtheria toxin (11). These chimeric toxins are expressed in *E. coli* and are specifically cytotoxic to the appropriate receptor-bearing cells. Having successfully made several different fusion proteins which recognize a single receptor or a single antigen on a cell, we wanted to construct a bifunctional molecule which would interact with two different cell surface receptors. The uses of such molecules will be described below. Here we report the construction of a chimeric molecule in which a cDNA for TGFα has been fused to the DNA of anti-Tac(Fv)-PE40. The protein produced by this gene fusion is termed TGFα-anti-Tac(Fv)-PE40 and selectively kills cells bearing either EGF or IL2 receptors but has low activity on cells without these receptors.

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Materials and Methods

Plasmid constructions: A plasmid for the expression of TGFα-anti-Tac(Fv)-PE40 using the T7 promoter was constructed with plasmids pJB70548 and pVC320. Plasmid pJB70548 carries a cDNA for the variable light chain of anti-Tac fused through a peptide linker to the variable heavy chain which is in turn attached to DNA for PE40. pJB70548 whose construction will be described elsewhere (Batra, Chaudhary and Pastan) is different than pVC70108 (4) in the orientation of the variable domains of anti-Tac cDNA. The linker used in this construction is also different. pJB70548 was linearized by restriction with NdeI. cDNA for TGFα was isolated from pVC320 by digestion with NdeI. The TGFα cDNA was subcloned as NdeI insert into the NdeI site of pJB70548. The resulting plasmid pJB37058 was examined for its size, orientation of TGFα and expression of TGFα -anti-Tac(Fv)-PE40 in BL21 (λDE3) cells (Fig. 1).

Expression and purification of the fusion protein: For expression and localization of the chimeric protein, TGFα–anti-Tac(Fv)-PE40, BL21 (λDE3) cells were transformed with the plasmid pJB370548 and cultured in Superbroth containing 100 μg/ml ampicillin. At OD_{650} of 0.8, the culture was induced with 1mM IPTG for 90 minutes. The cell pellet was further processed as described before (4).

Inclusion bodies were used as the source for purification of the fusion protein. After denaturation and renaturation of the toxic protein, the sample was applied on a FPLC Mono Q (10/10) column in 0.02 M Tris-HCl buffer pH 7.4. A linear gradient of 0-0.5 M NaCl was applied to elute the proteins from the column. Fractions were assayed for their ability to inhibit protein synthesis on HUT102 and A431 cells. Active fractions were pooled and used for further characterization.

Gel electrophoresis and immunoblotting: SDS/PAGE was done by the method of Laemmli using Bio-rad mini Protein II assembly (12). The gels were stained with Coomassie blue. For immunoblotting, electrophoresed samples were transferred onto nitrocellulose paper, which was then incubated with antibodies to native PE or human TGFα.

Protein synthesis inhibition assay: Activity of TGFα anti-Tac(Fv)PE40 was assayed on A431 and HUT102 cells by measuring the ^3H leucine incorporations as described (4). A431 is a human epidermoid carcinoma cell line and expresses EGF receptor whereas HU102 is a human T cell leukemia cell line and expresses interleukin 2 receptors. HUT102 cells were washed twice with RPMI 1640 and used immediately. A431 cells were seeded at 1×10^5/ml in 24 well plates 24 hrs before the addition of toxin. [^3H]-leucine incorporation into protein was measured 16-20 hours later (4).

Results and Discussion

The structure of a plasmid encoding TGFα-anti-Tac(Fv)-PE40 is shown in Figure 1. The assembled gene is under the control of a bacteriophage T7 promoter. The plasmid expresses the first 50 amino acids of mature TGFα, the first 106 amino acids of anti-Tac light chain, a 14 amino acid linker, the first 116 amino acids of anti-Tac heavy chain, and amino acids 253-613 of PE. To prepare the recombinant protein, plasmid pJB370548 was expressed in BL21 (λDE3) and a fusion protein of expected size (70 kDa) was produced. Because TGFα-anti-Tac(Fv)-PE40 was contained in the inclusion bodies, these were used to prepare protein (4). Following denaturation and renaturation, the chimeric toxin was applied on a mono Q ion exchange column and eluted from the column at 0.2-0.22 M NaCl (Fig. 2). Active fractions were detected by assaying inhibition of protein synthesis of HUT102 and A431 cells (Fig. 2). The active fractions were pooled and used for characterization of the chimeric toxin. Plasmids expressing TGFα-PE40 and anti-Tac(Fv)-PE40 were expressed in the same manner and the proteins purified by Mono Q chromatography. Fig. 3A, lane 1, shows a Coomassie

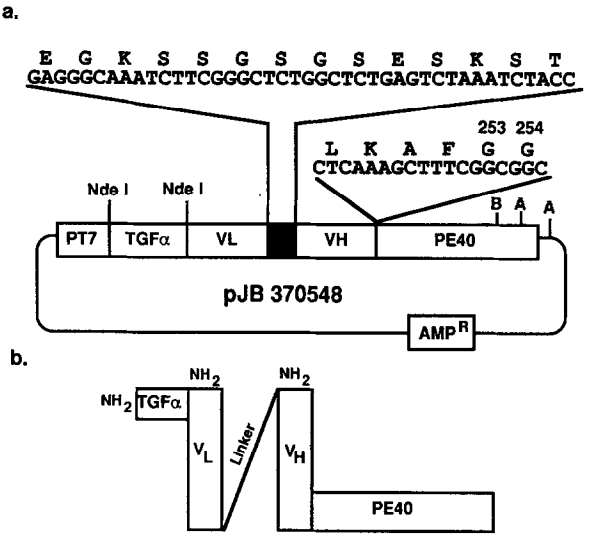

Figure 1: Expression vector for TGFα-anti-Tac(Fv)-PE40.

a. Expression plasmid pJB370548 contains a fusion gene encoding the first 50 amino acid of TGFα, the variable domain of anti-Tac light chain (VL, first 106 amino acids of anti-Tac light chain), a 14 amino acid linker (■), the variable anti-Tac heavy chain (VH, first 116 amino acids of heavy chain), and amino acids 253-613 of PE. AmpR, β-lactamase gene; B, BamH1; A, AvaI. Amino acids are shown as single letter codes. The gene is under the control of the bacteriophage T7 promoter linked to a Shine-Dalagarno sequence and an initiation codon.

b. Schematic arrangement of various domains of TGFα-antiTac(Fv)PE40.

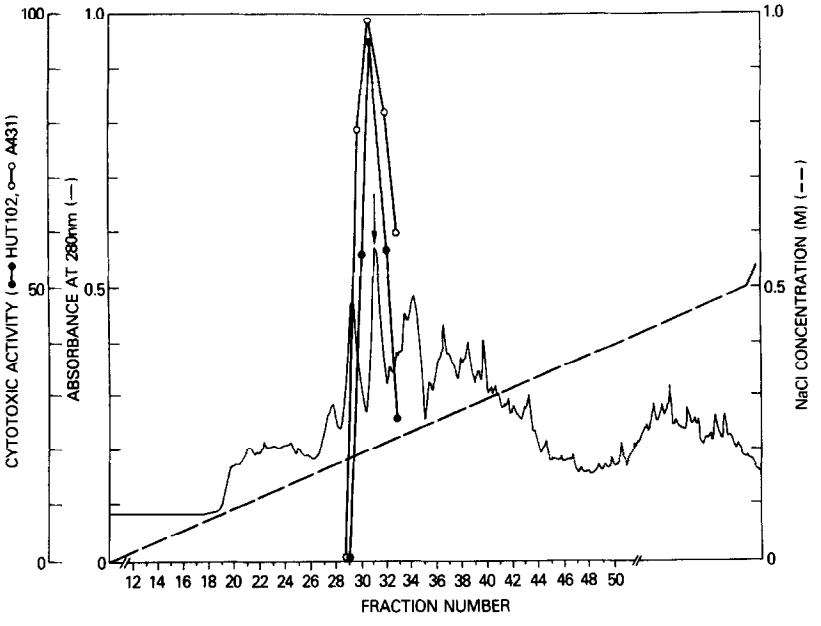

Figure 2: Partial purification of TGFα-anti-Tac(Fv)-PE40.

Inclusion bodies were used to purify the fusion protein. After denaturation and renaturation, the toxin was applied on a FPLC Mono Q (HR 10/10) column (4). A linear gradient of 0-0.5 M NaCl (---) was applied to elute the proteins which were monitored at 280 nm (-); 4.0 ml fractions were collected. The position of active fractions is indicated. Inhibition of protein synthesis on HUT 102 (●–●) and A431 cells (○–○) was measured (4). A pool of active fractions was used for further characterization.

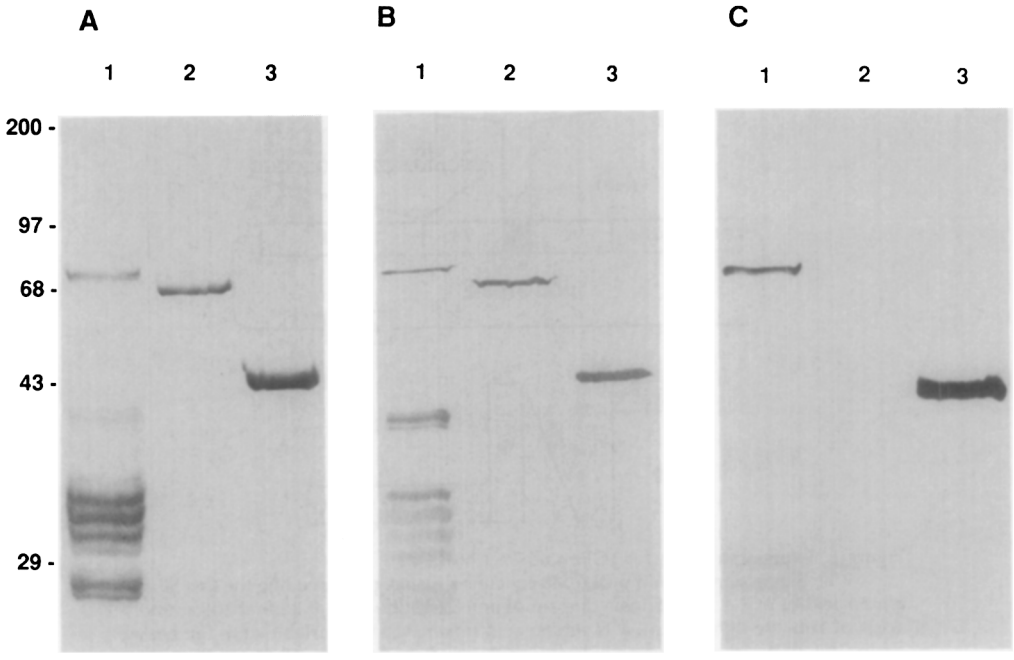

Figure 3: SDS-PAGE and immunoblots of TGFα-anti-Tac(Fv)-PE40. 10% SDS gels were run according to the method of Laemmli (12). A. Gel stained with Coomassie blue. B. Immunoblotting with antibody to PE. C. Immunoblotting with antibody to human TGFα. Lane 1, TGFα-anti-Tac(Fv)-PE40, Mono Q pool (fxn 31,32). Lane 2, anti-Tac(Fv)-PE40 Mono Q purified. Lane 3, TGFα-PE40 Mono Q purified. Molecular weight markers are indicated in kDa. Plasmids pJB70548 and pVC387 (7) were used for the expression of anti-Tac(Fv)-PE40 and TGFα-PE40, respectively.

blue stained gel of the partially purified TGFα anti-Tac(Fv)-PE40 which contains a band of the expected molecular weight (70 kDa) as well as several low molecular weight contaminants. TGFα-PE40 and anti-Tac(Fv)-PE40 were purified in a similar manner (Fig. 3A, lanes 2 and 3). Immunoblots with antibody to TGFα confirmed the presence of TGFα in the bifunctional chimeric protein (Fig. 3C) and in TGFα–PE40. The cytotoxicity of partially purified TGFα-anti-Tac(Fv)-PE40 was tested on A431 and HUT102 cells which express EGF and interleukin 2 receptors, respectively. Its activity was compared with that of TGFα-PE40 and anti-Tac(Fv)-PE40.

As shown in Fig. 4A and B, TGFα-anti-Tac(Fv)-PE40 inhibited protein synthesis on A431 and HUT102 cells in a dose-dependent manner. The cytotoxic effect was competed with excess EGF on A431 cells and excess anti-Tac on HUT102 cells showing the specific killing of these cells by the fusion protein. Anti-Tac(Fv)-PE40, used as a control, inhibited protein synthesis on HUT 102 cells but had no effect on A431 cells (Fig. 4C). TGFα-PE40 was cytotoxic to A431 cells and not to HUT102 cells (Fig. 4D).

The comparative activities of TGFα-anti-Tac(Fv)-PE40, TGFα-PE40 and anti-Tac(Fv)-PE40 after purification on Mono Q columns are shown in Table 1. TGFα-anti-

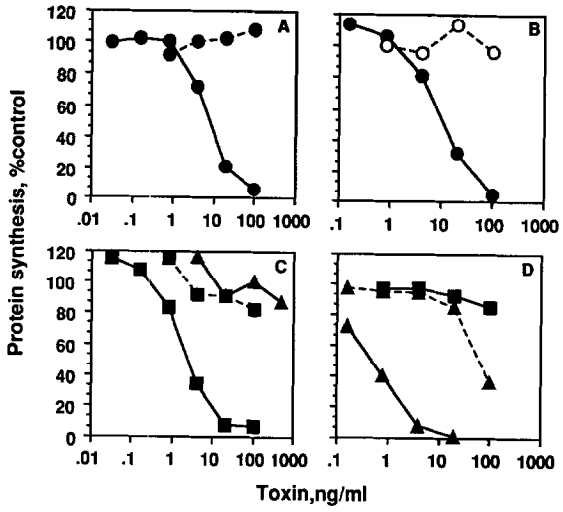

Figure 4: Cytotoxicity of TGFα-anti-Tac(Fv)-PE40 on HUT-102 and A431 cells.

Cytotoxic activity of the fusion protein was assayed on HUT-102 (A,C) and A431 (B,D) cells by measuring inhibition of protein synthesis as previously described (4).

TGFα-anti-Tac(Fv)-PE40 was added in the absence (●—●) and presence of either anti-Tac on HUT102 cells, (•···•) or EGF, 4 μg/ml on A431 cells (o--o) for 16-20 hrs. at 37°C. Cytotoxicity and specificity was compared to that of anti-Tac(Fv)-PE40 and TGFα-PE40 at the same time. anti-Tac(Fv)-PE40 alone (■—■), anti-Tac(Fv)-PE40 + 10 μg anti-Tac (■···■), TGFα-PE40 alone (▲—▲) and TGFα-PE40 + 4 μg EGF (▲--▲). Results are indicated as percent of control.

Tac(Fv)-PE40 inhibits protein synthesis on HUT102 and A431 cells with ID_{50}'s of 8 and 12 ng/ml, respectively. These cytotoxic activities are several-fold lower than the activity of TGFα-PE40 on A431 cells and anti-Tac(Fv)-PE40 on HUT 102 cells. The lower activity of TGFα-anti-Tac(Fv)-PE40 preparation is in part due to its relatively low purity. If the activity is calculated on the basis of the 70,000 Mr species, the preparations would be at least five times more active. The specific activity of anti-Tac(Fv)-PE40 shown here is lower than the value reported earlier (4) because of the presence of low molecular weight peptides that react with the Bradford reagent used to measure total protein. Further purification by gel filtration has been found to increase the specific activity of all the fusion proteins (4).

Table 1

Comparison of cytotoxic activity of bifunctional and monofunctional recombinant fusion proteins

	ID_{50}[a] (ng/ml)	
	HUT102	A431
TGFα-anti-Tac(Fv)-PE40	7.8	12.0
Anti-Tac(Fv)-PE40	2.3	~500
TGFα-PE40	>500	0.5

[a]ID_{50} is the concentration of the fusion protein that gave 50% inhibition of protein synthesis. Other details are described in Figure 4.

TGFα-anti-Tac(Fv)-PE40 is a bifunctional toxin that is active on cells with either EGF or IL2 receptors. Cytotoxic agents that bind to two different receptors or antigens might be extremely useful in killing cells more efficiently by taking advantage of two different cell surface receptors which are both efficiently internalized, or by using one receptor for binding and the other for promoting internalization in those cases where the former is not efficiently internalized. It also should be possible to create molecules in which the same ligand is present in two or more copies so that the binding to cell surface receptors is increased. In growth factors such as TGFα, interleukin 2, as well as in single chain antibodies, binding to cell surface receptors or antigens can still occur when the amino or carboxyl ends are modified by polypeptide extension. Therefore, it should be possible to make multivalent chimeric toxins containing the same or several different ligands.

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