Published in final edited form as:

Pancreas. 2012 July ; 41(5): 789–796. doi:10.1097/MPA.0b013e31823b5f2e.

A deimmunized bispecific ligand directed toxin that shows an impressive anti-pancreatic cancer effect in a systemic nude mouse orthotopic model

Seunguk Oh, PhD,

Department of Therapeutic Radiology, University of Minnesota Cancer Center, Minneapolis, Minnesota

Deborah A. Todhunter, PhD,

Department of Therapeutic Radiology, University of Minnesota Cancer Center, Minneapolis, Minnesota

Angela Panoskaltsis-Mortari, PhD,

Department of Pediatrics, University of Minnesota Cancer Center Minneapolis, Minnesota

Donald J. Buchsbaum, PhD,

Department of Radiation Oncology, University of Alabama at Birmingham Birmingham, Alabama

Shoko Toma, M.S., and

Department of Therapeutic Radiology, University of Minnesota Cancer Center, Minneapolis, Minnesota

Daniel A. Vallera, PhD

Department of Therapeutic Radiology, University of Minnesota Cancer Center, Minneapolis, Minnesota

Abstract

Objective—The objective was to test a bispecific ligand directed toxin (BLT), with reduced immunogenicity for enhanced efficacy in targeting orthotopic pancreatic cancer in vivo.

Method—A new BLT was created in which both human EGF and IL-4 cytokines were cloned onto the same single chain molecule with deimmunized pseudomonas exotoxin (dEGF4KDEL). Key amino acids dictating B cell generation of neutralizing anti-toxin antibodies were mutated. Bioassays were used to determine whether mutation reduced potency, and ELISA studies were performed to determine whether anti-toxin antibodies were reduced. A genetically altered luciferase MIA PaCa-2 xenograft model was used to image in real time and determine effects on systemic malignant human cancer. BLTs targeting B cells were used as specificity controls.

Results—dEGF4KDEL was significantly effective following systemic injection against established orthotopic MIA PaCa-2 pancreatic cancer and selectively prevented metastasis. Mutagenesis significantly reduced anti-toxin levels in vivo with no apparent activity loss in vitro. The drug was effective against three human pancreatic cancer lines in vitro, MIA PaCa-2, SW1990, and S2VP10.

Corresponding author DAV: MMC:367 Minneapolis, MN 55455 valle001@umn.edu, phone 612-626-6664, fax 612-624-4842.

Disclosure Statement: No potential conflicts of interest were identified in association with this work including financial interests or affiliations with institutions, organizations, or companies. No financial associations involving spouses, partners, or children were identified.

Conclusions—Despite the metastatic nature of the MIA PaCa-2 orthotopic tumor xenografted in nude mice, high percentages of tumors responded to extended dEGFKDEL treatment resulting in significant anti-cancer effects and disease-free survivors.

Keywords

immunotoxin; pancreatic cancer; xenograft model; EGF; pseudomonas exotoxin; immunoconjugate therapy

Introduction

Pancreatic cancer has a poor prognosis and surgery is effective in only 10–15% of the cases. Thousands die each year because of rapid growth and metastasis (1, 2). Despite drug therapy, the median survival time is measured in months (3, 4). Pancreatic tumors are known to overexpress epidermal growth factor receptor (EGFR), a transmembrane signaling protein from the erbB family (5, 6). Studies have revealed a link between EGFR signaling pathways and malignancy (7). Therefore, fusion toxins targeting EGFR show promise for pancreatic cancer therapy (8–11). In some of the earlier work, targeted toxins directed by transforming growth factor- α (TGF- α) exerted growth inhibitory effects on cells bearing a high number of EGF receptors (12). Interleukin 4 receptor (IL-4R) is another putative pancreatic cancer target and is expressed on pancreatic tumors. For example, investigators showed that 6 of 6 pancreatic cancer cell lines examined expressed IL-4R (13) and that IL-4R is internalized at high levels after binding to IL-4 (14, 15). IL-4R-directed fusion toxins have also been shown to kill pancreatic tumor cells (14). IL-4R is an excellent target because its normal expression is mostly limited to hematopoietic cells.

In this study, we examined a novel single-chain recombinant bispecific ligand directed toxin (BLT) by linking human EGF and genetically altered IL-4 to a fragment of deimmunized Pseudomonas exotoxin (PE38). PE catalyzes ADP ribosylation of elongation factor 2 (EF-2) leading to irreversible inhibition of protein synthesis and cell death at very low concentrations (16).

One major shortcoming of this class of drugs is the immunogenicity of the toxin (17–19). Multiple treatments with drug are necessary to cause tumor regressions and this results in the generation of anti-toxin antibodies that block drug efficacy. Onda and Pastan recently mapped seven major immunodominant epitopes in PE38 that can be mutated without loss of toxin activity (18,19). Thus, we used this advancement to produce a new deimmunized anti-pancreatic cancer biological drug.

In this study, deimmunized EGF4KDEL (dEGF4KDEL) was bioengineered and compared to its monospecific counterparts *in vitro*. The superior anti-proliferative activity against the human pancreatic cell line MIA PaCa-2 translated into a potent *in vivo* anti-pancreatic cancer effect in a systemic, orthotopic, pancreatic cancer xenograft model in which tumor growth could be monitored in real time. The fact that this agent is deimmunized allowing for multiple treatments renders it an improvement over our earlier attempts at BLT development.

Material and Methods

Construction of EGF4KDEL and dEGF4KDEL

DNA shuffling and PCR assembly techniques were used to assemble the genes encoding the single chain bispecific immunotoxin EGF4KDEL. The fully assembled fusion gene (from 5' end to 3' end) consisted of an NcoI restriction site, an ATG initiation codon, the genes for

human EGF and circularly permuted human IL-4 linked by a 20 amino-acid segment of human muscle aldolase (HMA), the 7 amino-acid EASGGPE linker, the first 362 amino acids of the PE molecule with KDEL replacing the REDLK at the c-terminus, and a *Not I* restriction site at the 3' end of the construct (Figure 1). The HMA segment was incorporated into the molecule as a flexible, non-immunogenic linker (20). The gene-encoding circularly permuted IL-4 was generously provided by Dr. R.J. Kreitman and Dr. I Pastan (NIH, Bethesda MD, USA). The resultant 1752 bp *NcoI/NotI* fragment gene was spliced into the pET21d bacteria expression vector under control of an isopropyl-b-D-thiogalactopyranoside (IPTG) inducible T7 promoter. The gene was cloned and verified by sequencing (Biomedical Genomics Center, University of Minnesota). The monospecific agents, EGFKDEL and IL4KDEL were created using the same techniques. To create a deimmunized EGF4KDEL, eight amino acids representing the seven major epitopes on PE38 (18) were mutated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla CA, USA). The following amino acids were altered and verified: R490A, R513A, R467A, E548S, K590S, R432G, Q332S, R313A. Additional BLTs targeting hematological malignancies were assembled and used as negative controls in this study. CD3CD3KDEL, a negative control, consisting of two repeating scFvs targeting human CD3 was made by replacing the DT390 portion of the DTCD3CD3 molecule described previously (21), with PE38KDEL. Deimmunized 2219ARLKDEL, a negative control recognizing human B cells, was produced by joining two scFvs specific for human anti-CD22 and anti-CD19 to the same deimmunized PE38KDEL used in dEGF4KDEL (22).

Isolation of inclusion bodies, refolding and purification

Proteins were produced as described previously (23) with some minor modifications to improve yield and purity. Then, 10 mg/ml of dithiothreitol was included in refolding buffer to decrease protein aggregation. In addition, refolded protein was directly diluted instead of being dialyzed before loading onto an ion exchange column. Finally, the purity of protein isolated from the ion exchange column was further enhanced using an FPLC and Superdex 200 HiLoad 26/60 size exclusion column (Sigma, Ronconcoma, NY, USA). This modified protocol resulted in a yield of 5 – 10 mg of protein per liter of culture and a final product with 95% purity.

Cell culture

The human malignant pancreatic cell lines MIA PaCa-2, S2VP10, SW1990 (24) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cell lines were maintained in DMEM (MIA PaCa-2) or RPMI-1640 (S2VP10, SW1990) (Cambrex, East Rutherford, NJ, USA) supplemented with 10% fetal bovine serum, 2 mmol/LL-glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin. Cell cultures were incubated in a humidified 37°C atmosphere containing 5% CO₂. When adherent cells were 80–90% confluent they were passaged using trypsin-EDTA for detachment. Only cells with viability >95%, as determined by trypan blue exclusion, were used for experiments.

For *in vivo* experiments, MIA PaCa-2 cells were stably transfected with dual vectors containing both the firefly luciferase (*luc*) and green fluorescent protein (GFP) genes, as well as a Blastocidin resistance gene (Clontech Laboratories, Mountain View, CA, USA). Transfection was performed with Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) and stable clones were established using a FACS Diva flow cytometer (University of Minnesota Flow Cytometry Core Facility of the Masonic Cancer Center) to seed individual GFP-positive cells in a 96-well plate. MIA PaCa-2-*luc* retained identical morphological and biological properties to the specific parental cell line and was maintained with additional 10 µg of Blastocidin (InvivoGen, San Diego, CA).

Bioassays to measure in vitro protein synthesis inhibition

To determine the effect of EGF4KDEL and dEGF4KDEL on pancreatic cancer cell lines, a proliferation assay measuring ^3H -thymidine incorporation was used. Cells (10^4 per well) were added to a 96-well flat-bottomed plate and incubated overnight at 37°C with 5% CO_2 to allow cells to adhere. The BLTs in varying concentrations were added to wells in triplicate. Incubation at 37°C and 5% CO_2 continued for 72 hours. [Methyl- ^3H]-thymidine (GE Healthcare, UK) was added ($1\ \mu\text{Ci}$ per well) for the final 8 hours of incubation. Plates were frozen to detach cells and cells were then harvested onto a glass fiber filter, washed, dried, and counted using standard scintillation methods. Data are reported as a percentage of control counts. For blocking studies, increasing concentrations of EGF4 were mixed with $1\ \text{nM}$ EGF4KDEL. Subsequent mixtures were added to wells containing MIA PaCa-2 cells. All other aspects of blocking assays were identical to the procedure listed above for proliferation assays.

In some cases, bioactivity was similarly assessed using ^3H -leucine incorporation assay (22). The procedure was identical with the thymidine proliferation assay except leucine-free media (Caisson Labs, North Logan, UT, USA) was used and the incubation period after ^3H -leucine (GE Healthcare, Chalfont St Giles, UK) addition ($1\ \mu\text{Ci}$ per well) was longer (24 hrs). Data collection and analysis proceeded as described above. CD3CD3KDEL, a recombinant bispecific scFv targeting T cells was used as a negative control to establish selectivity.

Determining immunogenicity of EGF4KDEL and dEGF4KDEL in BALB/c mice

Mouse immunization studies were used to determine whether mutated dEGF4KDEL elicited less of an immune response than non-mutated parental EGF4KDEL. Female BALB/c mice ($n=5/\text{group}$) were injected intraperitoneally once weekly with $0.25\ \mu\text{g}$ of either EGF4KDEL or dEGF4KDEL for 63 days. Each week, five days after injection, the mice were bled (facial vein collection) to obtain serum. Serum from each mouse was isolated using centrifugation and frozen. Levels of anti-PE38KDEL IgG in each serum sample was measured using ELISA. Briefly, $5\ \mu\text{g}$ of purified recombinant PE38KDEL was added to each well of a 96-well microtiter plate and adhered overnight at 4°C . Unbound protein was washed away with PBS-T and blocking was performed for 1 h with 5% milk/PBS-T. Serum samples were diluted in 1:10,000 and $100\ \mu\text{l}$ of each was added to appropriate wells in triplicate. Following 3 hrs incubation, each well was washed 3 times with PBS-T. Peroxidase-conjugated rabbit anti-mouse IgG (Sigma) was added to each well for 2 hrs room-temperature incubation. After washing, o-phenylenediamine dihydrochloride substrate was added to each well. After 30 min, the absorbance at 490 nm was measured using a microplate reader. Quantification of actual anti-PE38KDEL IgG present in each sample was determined by comparing the absorbance values in each well to a standard curve prepared using M40-1 monoclonal anti-PE38KDEL antibody from Dr. Robert Kreitman (NIH, Bethesda, MD).

In vivo efficacy of EGF4KDEL and dEGF4KDEL against orthotopic pancreatic cancer xenograft model

Male nu/nu mice or normal mice were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center, Animal Production Area and housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited specific pathogen-free facility under the care of the Department of Research Animal Resources, University of Minnesota. Animal research protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee. All animals were housed in microisolator cages to minimize horizontal pathogen contamination.

For orthotopic inoculation of MIA PaCa-2-luc cells, mice were anesthetized with 14 μ l/g of ketamine/xylazine cocktail (ketamine 6.5 mg/ml, xylazine 0.44 mg/ml). A small 0.5 cm incision was made in the left abdomen and 4×10^6 cells were injected orthotopically into the exposed pancreas (1:1 mixture cell:matrigel (BD bioscience, San Jose, CA)) using a 29 gauge needle and a Hamilton syringe. In some experiments, dye was injected with cells in extra mice to confirm that cells were not leaking from the injection site. The peritoneum was then closed with dissolvable suture and the skin incision closed with wound clips (Stoelting Co, Wood Dale, IL).

The effect of dEGF4KDEL against the MIA PaCa-2-luc orthotopic tumors was measured using bioluminescent imaging in real time. Mice were imaged every week to monitor the level of luciferase activity and tumor progression. Images were captured using the Xenogen IVIS 100 imaging system and analyzed with Living Image 2.5 software (Xenogen Corporation, Hopkington, MA). During imaging, mice were lightly anesthetized with isoflurane gas. All mice received 100 μ l of a 30 mg/ml D-luciferin aqueous solution (Gold Biotechnology, St. Louis MO) IP 10 minutes before imaging to provide a substrate for the luciferase enzyme. All images represent 5 minutes exposure time and all regions of interest (ROI) are expressed in units of photons/sec/cm²/sr.

For *in vivo* studies, athymic nude mice were injected orthotopically (OT) with 4 million MIA PaCa-2-luc cells on day 0 to initiate tumors and given 3–4 μ g of dEGF4KDEL/ IP injection. For Experiment 1, a single course of treatment was one injection given every other day (MWF). Treatment began on day 5 and mice were given 9 weekly courses of dEGF4KDEL IP of about 4 μ g/injection. In experiment 2, mice were treated using a different schedule. A single course of drug treatment was 2 injections given every day (MTWTh). Treatment began on day 3 and mice were given 3 μ g/injection with 7 courses. In experiment 3, a single course of treatment was 1 injection given every day (MTWTh). Treatment began on day 5 and mice were given 4 μ g/injection for 7 weekly courses. Bioluminescent images were carried out to monitor the response to treatment every week. If weight loss was detected, an injection was occasionally reduced or skipped.

Histology

Mice from *in vivo* experiments were sacrificed after bioluminescent imaging for histologic evaluation. For histology, myocardial perfusion was performed. Following this, tissues were removed and embedded in OCT compound (Miles, Elkhart, IN), snap frozen in liquid nitrogen, and stored at -80°C until sectioned. Sections were cut, thawed, and mounted on glass slides and fixed for 5 minutes in acetone. Slides were stained with hematoxylin and eosin (H & E).

Statistical analyses

All statistical analysis was performed using Prism 4 (Graphpad Software, San Diego, CA, USA). Groupwise comparisons of single data points were made by Student's t-test. P-values <0.05 were considered significant.

Results

Potency and specificity of EGF4KDEL and dEGF4KDEL

EGF4KDEL was column purified to a purity of 95% (not shown) and then tested against the EGFR+IL-4R+ human pancreatic cancer cell line MIA PaCa-2. The MIA PaCa-2 cell line was chosen because it grows well in nude mice and orthotopic injection always results in pancreatic cancer and liver metastasis. Figure 2 shows that bispecific EGF4KDEL (with an IC₅₀ of 5×10^{-7} nM) was at least 2 logs more effective in inhibiting the proliferation of

MIA PaCa-2 *in vitro* than its monospecific counterparts. Also, EGF4KDEL was at least 2 logs more effective than an equimolar mixture of monospecific EGFKDEL and cpIL4KDEL indicating that the superior effect was dependent on positioning both ligands on the same single chain molecule.

In order to determine whether EGF and IL-4 ligands bound to EGFR+ IL4R+ target cells, recombinant EGF4 devoid of toxin was synthesized and added to wells containing 1 nM of EGF4KDEL and MIA PaCa-2 target cells (Figure 3). The addition of EGF4 blocked the ability of EGF4KDEL to kill target cells. In other studies, both anti-EGF and anti-IL-4 antibodies blocked EGF4KDEL activity indicating that both ligands were individually active (25, 26). In Figure 4A, dEGF4KDEL was tested against EGFR+IL-4R+ SW1990 human pancreatic cancer cells in proliferation assays. dEGF4KDEL was highly inhibitory with an IC₅₀ of 10⁻⁵ nM, while irrelevant control targeted toxin CD3CD3KDEL had minimal effect. In Figure 4B, deimmunized EGF4KDEL was compared to non-mutated parental EGF4KDEL against another pancreatic cancer S2VP10 cell line. The mutated drug displayed an IC₅₀ of 10⁻⁹ nM and the activity of the two drugs was identical. Activities were similar when mutated and non-mutated drug were also compared against the MIA PaCa-2 and SW1990 cell lines (not shown). Together, these data indicate the drugs were potent and highly selective against pancreatic carcinoma cells. Also, mutation of toxin did not have any effect on activity of the drug.

Efficacy in an aggressive orthotopic pancreatic cancer model

To determine whether deimmunized drug could mediate a systemic anti-pancreatic cancer effect, a model was developed in which MIA PaCa-2 cells transfected with a luciferase reporter gene were surgically injected into the pancreas of nude mice. In all three experiments shown in Figure 5, animals received the same number of MIA PaCa-2 cells by OT injection. All drug treatments were 3–4 µg/injection by IP injection. In Figure 5, Experiment 1, animals were treated with a single course of drug at 4ug/injection given every other day (MWF) beginning on day 5. Mice were given a total of 9 weekly courses of treatment. The individual data from all animals M8–M11 show a marked reduction of tumor over time in 4 of 5 treated mice as determined by a reduction in total bioluminescence activity. These animals survived long-term (172 days) and animals M8 and M10 were tumor-free survivors. Tissues were examined for the presence of metastatic cancer using standard histology techniques and tumor cells could not be detected. In contrast, untreated controls (M1–M7) mostly progressed.

In Figure 5, Experiment 2, tumor-bearing mice were treated with deimmunized drug using a different schedule. A single course of drug treatment was two 3 ug injections given every day (MTWTh) beginning on day 3. Mice were given a total of 7 weekly courses. The results were similar with regressions in 4 of 6 treated mice. Mice M34–M37 were still tumor free after 67 days as determined by histology analysis. The experiment was repeated in Figure 5, Experiment 3. This time animals were treated on MTWTh with a single IP injection of drug (4 ug/injection) beginning on day 5. Again mice were treated with 7 weekly courses. In four of 5 treated animals tumors regressed, but all animals relapsed by day 54.

Figure 6 shows the average tumour-associated luciferase activity from animals in each group of the mice in Experiment 1, measured by plotting total bioluminescent activity over time. The data are plotted for the 4 animals that responded to drug. A group of 3 mice were given deimmunized 2219ARLKDEL as a control since it only recognizes B cells. Tumor growth paralleled that observed in the untreated controls. Taken together, these data show that deimmunized drug is highly effective and selective against systemic human pancreatic cancer and the best results were observed with extended treatment with drug.

Because imaging indicated that mice M8–M11 that were treated with dEGF4KDEL were tumor-free on day 172, histology studies of liver, kidney, spleen, and pancreas were performed. M8 was studied for histology on day 201 and was tumor free (data not shown). For Experiment 2, mice M35 and M37 were tumor free three weeks after the completion of the experiment by the image results. Despite the extended treatment, the M35 histology panel in Figure 5 reveals a normal hepatic lobule and no inflammation around the portal vein signifying a healthy liver. The M37 histology panel figure shows a healthy pancreas including a healthy lobular exocrine duct (right) and islet of Langerhans (left). Histology studies were also performed on mice with OT tumors that were not treated. The histology panel of mouse M25 pancreas revealed a small area of inflammatory infiltrate containing tumor cells. The histology panel of mouse M26 showed advanced pancreatic infiltration by the tumor cells and deterioration of the pancreatic architecture. This correlated with the imaging data which showed a focus of bioluminescent activity.

“Reduced immunogenicity” of dEGF4KDEL

To decrease the immunogenicity of EGF4KDEL, B-cell epitopes on the PE38(KDEL) portion were mutated. Immunization studies with immunocompetent BALB/c mice were conducted to compare the immunogenicity of deimmunized EGF4KDEL to the parental EGF4KDEL molecule. Mice (n=5/group) were immunized weekly for a total of 9 weeks. Serum samples from each animal were analyzed using ELISA to detect anti-PE38(KDEL) IgG. Figure 7 shows that mice injected with the original EGF4KDEL agent showed a steady increase in anti-toxin levels as more injections were given. The levels of anti-toxin antibody also rose for dEGF4KDEL, but at a reduced rate. Despite multiple immunizations, levels of anti-toxin were significantly reduced with the administration of deimmunized drug ($p<0.05$).

Discussion

The original contribution of this paper is that a BLT called dEGF4KDEL simultaneously targeting EGFR and IL-4R had striking effects against systemic human pancreatic cancer in an orthotopic nude mouse model. Despite the metastatic nature of the MIA PaCa-2 orthotopic tumor xenografted in nude mice, high percentages of the tumors responded resulting in a significant anti-cancer effect and disease free survivors. Multiple injections of dEGF4KDEL were tolerated and a therapeutic window exists despite the reactivity of human EGF with the native receptors in the mouse. Different regimens were tested and all showed some degree of efficacy, but we were unable to conclude that one regimen was better than another. However, our data in total does indicate that deimmunized drug is highly effective and selective against orthotopic pancreatic cancer and extending treatment is important in obtaining long-term survivors. The BLT had greater activity than its monospecific forms and studies with mixtures of the monomeric forms, EGFKDEL and IL4KDEL, showed that the enhanced activity was dependent on having both ligands on the same single chain molecule. Activity was selective because negative controls did not cause an anti-cancer effect. The drug was highly effective against three different pancreatic carcinoma cell lines.

The relevancy of targeted toxins has been rightfully criticized because of their diminished potency, immunogenicity, and toxicity. However, genetic engineering shows great potential to address all of these issues. To enhance potency, our laboratory used a drug framework permitting simultaneous delivery of targeted toxin with dual ligands. Our first constructs used anti-B cell scFVs and were based on the work of Herrera (27) that showed advantages of combining anti-CD19 and anti-CD22 based drugs to promote activity. Our studies showed that BLTs were superior to the monospecific forms and to mixtures of these monospecific drugs indicating the importance of positioning both ligands on the same single chain molecule as the toxin. In subsequent studies, we found that cytokine ligands could be

substituted for scFV ligands to target carcinomas and that EGF and IL-4 ligands showed enhance potency in targeting aggressive carcinomas including breast and lung cancer (25, 26).

To address the issue of toxin immunogenicity, the toxin moiety was “deimmunized” without compromising its activity. The clinical efficacy of treatment with targeted toxins against solid tumors hinges on the ability to give multiple treatments (25). Anti-toxin responses will be generated that significantly reduce drug efficacy over time causing problems in two ways. 1) Neutralizing antibodies block drug efficacy (28) and 2) Non-neutralizing antibodies bind to drug and accelerate clearance, negatively impacting its pharmacokinetics (29, 30). Onda and Pastan used a library of anti-PE monoclonal antibodies to epitope map prominent molecular region, which elicited the strongest antibody response from immunized B cells (18). PE was mapped into seven epitope regions. We constructed our PE-based BLT and mutated key amino acids in these 7 regions without compromising toxin activity. The anti-toxin response was significantly reduced in a validated mouse model (25). We are continuing to search for additional immunogenic amino acids that will further reduce immunogenicity.

A key question is: Is the mouse an acceptable model to study the immunogenicity of PE? To address this, investigators asked whether treatment with PE immunotoxins induces human antibody responses to the same immunogenic epitopes recognized by mice. Sera was taken from 8 patients in phase 1 trials with pancreatic, colon cancer, or mesothelioma treated with two different PE immunotoxins LMB-9 (anti-CD25 PE) (31) or SS1P (anti-mesothelin PE) (32). These patients had developed neutralizing antibodies that prevented further drug administration. Serum samples were analyzed in a published assay and most patients with solid tumors produced neutralizing Abs after one cycle of immunotoxin treatment (33). Competition analysis of paired serum samples showed that before treatment, the sera contained almost no specific Ab to any of the PE38 epitopes. In contrast, the sera obtained after immunotoxin treatment contained anti-PE38 Abs to every epitope recognized by the mouse antisera as shown by their ability to inhibit the binding of the corresponding mAbs to each epitope (17). These results show that human immunotoxin treatment induces human Abs against the same 7 immunogenic epitopes identified by the mouse mAb panel.

Toxicity is still a major concern. Clinically, other targeted toxins were limited by renal or hepatic toxicity (34, 35). Although we have observed dermatological effects of EGFR-containing BLT in nude mice (23), a finding in agreement with the known reactivity of EGF with EGFR in the skin (36), human IL-4 does not bind to murine IL-4R. Thus, mouse is not the best model for predicting toxicity in humans. Studies are underway to evaluate toxicity in an “ontarget” macaque model. Toxicity may be reduced in macaques by the higher molecular weight of the bispecific drug that might prevent kidney filtration and extend serum half-life.

Regarding the issue of whether genetic engineering can be used to reduce toxicity, a study was recently undertaken using this same orthotopic pancreatic cancer model in which the objective was to determine whether a method could be developed to block normal receptors while still targeting receptors over-expressed on cancer cells thereby decreasing toxicity while maintaining efficacy (37). A large bolus IP dose of genetically engineered EGF13 (devoid of toxin) was administered to mice about 15–20 minutes prior to DTEGF13, also given IP. Thus, the MTD was exceeded by 10–15 fold while still maintaining efficacy against systemic orthotopic pancreatic cancer in nude mice. Such an approach might be useful in this model as well.

Generally, the potency of BLTs is elevated over monospecific forms. However, sometimes EGF4KDEL is 1000 fold more active than either single ligand toxin EGFKDEL or IL4KDEL (25), and sometimes it is only marginally more active than a single ligand toxin (26). We believe the explanation is complex and influenced by more than one variable including the recognition and binding of two sets of receptors by the BLT, variances in receptor expression by the target cell, and differences in internalization by BLT versus single ligand toxins. Finally, it is well established that different targeted toxins compartmentalize differently within cells. For example, it is known that some are more prone to enter lysosomes and undergo degradation and some arrive rapidly to the cytosolic compartment to inhibit protein synthesis (38).

In conclusion, dEGF4KDEL represents a new anti-pancreatic cancer agent. Its bispecific construction is based on molecules that react with well-studied and established cancer targets, IL-4R and EGFR. *In vitro* studies show conclusive proof that the presence of both ligands on the same molecule is responsible for its superior activity. Possibly, the broader bispecific reactivity and lack of immunogenicity will be an advantage over earlier monospecific targeted toxins designed for use in pancreatic cancer (12). Our animal studies indicate that dEGF4KDEL is highly effective in checking aggressive tumor progression and reasonably well tolerated. Deimmunized EGF4KDEL may be a useful alternative therapy for pancreatic cancer and other types of EGFR+ carcinomas as well.

Acknowledgments

Support: This work was supported in part by the US Public Health Service Grants R01-CA36725, R01-CA082154, and P20 CA101955 awarded by the NCI and the NIAID, DHHS, the Randy Shaver Foundation, and the Hirshberg Award, American Pancreatic Association

References

1. Sener SF, Fremgen A, Menck HR, et al. Pancreatic cancer: a report of treatment and survival trends for 100,313 patients diagnosed from 1985–1995, using the National Cancer Database. *J Am Coll Surg*. 1999; 189:1–7. [PubMed: 10401733]
2. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. *CA Cancer J Clin*. 2006; 56:106–30. [PubMed: 16514137]
3. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2007. *CA Cancer J Clin*. 2007; 57:43–66. [PubMed: 17237035]
4. Tempero M, Plunkett W, Ruiz Van Haperen V, et al. Randomized phase II comparison of dose-intense gemcitabine: thirty-minute infusion and fixed dose rate infusion in patients with pancreatic adenocarcinoma. *J Clin Oncol*. 2003; 21:3402–8. [PubMed: 12885837]
5. Tzeng CW, Frolov A, Frolova N, et al. Epidermal growth factor receptor (EGFR) is highly conserved in pancreatic cancer. *Surgery*. 2007; 141:464–9. [PubMed: 17383523]
6. Lin CR, Chen WS, Kruiger W, et al. Expression cloning of human EGF receptor complementary DNA: gene amplification and three related messenger RNA products in A431 cells. *Science*. 1984; 224:843–8. [PubMed: 6326261]
7. Lo HW, Hung MC. Nuclear EGFR signalling network in cancers: linking EGFR pathway to cell cycle progression, nitric oxide pathway and patient survival. *Br J Cancer*. 2006; 94:184–8. [PubMed: 16434982]
8. Bruell D, Bruns CJ, Yezhelyev M, et al. Recombinant anti-EGFR immunotoxin 425(scFv)-ETA' demonstrates anti-tumor activity against disseminated human pancreatic cancer in nude mice. *Int J Mol Med*. 2005; 15:305–13. [PubMed: 15647848]
9. Bruell D, Stocker M, Huhn M, et al. The recombinant anti-EGF receptor immunotoxin 425(scFv)-ETA' suppresses growth of a highly metastatic pancreatic carcinoma cell line. *Int J Oncol*. 2003; 23:1179–86. [PubMed: 12964002]

10. Psarras K, Ueda M, Yamamura T, et al. Human pancreatic RNase1-human epidermal growth factor fusion: an entirely human 'immunotoxin analog' with cytotoxic properties against squamous cell carcinomas. *Protein Eng.* 1998; 11:1285–92. [PubMed: 9930679]
11. Jinno H, Ueda M, Ozawa S, et al. Epidermal growth factor receptor-dependent cytotoxic effect by an EGF-ribonuclease conjugate on human cancer cell lines--a trial for less immunogenic chimeric toxin. *Cancer Chemother Pharmacol.* 1996; 38:303–8. [PubMed: 8674151]
12. Baldwin RL, Kobrin MS, Tran T, Pastan I, Korc M. Cytotoxic effects of TGF-alpha-Pseudomonas exotoxin A fusion protein in human pancreatic carcinoma cells. *Pancreas.* 1996; 13:16–21. [PubMed: 8783329]
13. Kornmann M, Kleeff J, Debinski W, et al. Pancreatic cancer cells express interleukin-13 and -4 receptors, and their growth is inhibited by Pseudomonas exotoxin coupled to interleukin-13 and -4. *Anticancer Res.* 1999; 19:125–31. [PubMed: 10226533]
14. Kawakami K, Kawakami M, Leland P, et al. Internalization property of interleukin-4 receptor alpha chain increases cytotoxic effect of interleukin-4 receptor-targeted cytotoxin in cancer cells. *Clin Cancer Res.* 2002; 8:258–66. [PubMed: 11801567]
15. Friedrich K, Kammer W, Erhardt I, et al. The two subunits of the interleukin-4 receptor mediate independent and distinct patterns of ligand endocytosis. *Eur J Biochem.* 1999; 265:457–65. [PubMed: 10491204]
16. Jinno Y, Ogata M, Chaudhary VK, et al. Domain II mutants of Pseudomonas exotoxin deficient in translocation. *J Biol Chem.* 1989; 264:15953–9. [PubMed: 2506173]
17. Nagata S, Pastan I. Removal of B cell epitopes as a practical approach for reducing the immunogenicity of foreign protein-based therapeutics. *Adv Drug Deliv Rev.* 2009; 61:977–85. [PubMed: 19679153]
18. Onda M, Nagata S, FitzGerald DJ, et al. Characterization of the B cell epitopes associated with a truncated form of Pseudomonas exotoxin (PE38) used to make immunotoxins for the treatment of cancer patients. *J Immunol.* 2006; 177:8822–34. [PubMed: 17142785]
19. Onda M, Beers R, Xiang L, et al. An immunotoxin with greatly reduced immunogenicity by identification and removal of B cell epitopes. *Proc Natl Acad Sci USA.* 2008; 105:11311–6. [PubMed: 18678888]
20. Vallera DA, Todhunter DA, Kuroki DW, et al. A bispecific recombinant immunotoxin, DT2219, targeting human CD19 and CD22 receptors in a mouse xenograft model of B-cell leukemia/lymphoma. *Clin Cancer Res.* 2005; 11:3879–88. [PubMed: 15897589]
21. Vallera DA, Todhunter D, Kuroki DW, et al. Molecular modification of a recombinant, bivalent anti-human CD3 immunotoxin (Bic3) results in reduced in vivo toxicity in mice. *Leuk Res.* 2005; 29:249–51. [PubMed: 15661259]
22. Vallera DA, Oh S, Chen H, et al. Bioengineering a unique deimmunized bispecific targeted toxin that simultaneously recognizes human CD22 and CD19 receptors in a mouse model of B-cell metastases. *Mol Cancer Ther.* 2010; 9:1872–83. [PubMed: 20530709]
23. Vallera DA, Shu Y, Chen H, et al. Genetically designing a more potent anti-pancreatic cancer agent by simultaneously cotargeting human IL-13 and EGF receptors in a mouse xenograft model. *Gut.* 2008; 57:634–41. [PubMed: 18222985]
24. Yunis AA, Arimura GK, Russin DJ. Human pancreatic carcinoma (MIA PaCa-2) in continuous culture: sensitivity to asparaginase. *Int J Cancer.* 1977; 19:128–35. [PubMed: 832918]
25. Oh S, Stish BJ, Sachdev D, et al. A novel reduced immunogenicity bispecific targeted toxin simultaneously recognizing human epidermal growth factor and interleukin-4 receptors in a mouse model of metastatic breast carcinoma. *Clin Cancer Res.* 2009; 15:6137–47. [PubMed: 19789305]
26. Stish BJ, Oh S, Chen H, et al. Design and modification of EGF4KDEL 7Mut, a novel bispecific ligand-directed toxin, with decreased immunogenicity and potent anti-mesothelioma activity. *Br J Cancer.* 2009; 101:1114–23. [PubMed: 19755995]
27. Herrera L, Yarbrough S, Ghetie V, et al. Treatment of SCID/human B cell precursor ALL with anti-CD19 and anti-CD22 immunotoxins. *Leukemia.* 2003; 17:334–8. [PubMed: 12592332]
28. Vartanian TK, Zamvil SS, Fox E, et al. Neutralizing antibodies to disease-modifying agents in the treatment of multiple sclerosis. *Neurology.* 2004; 63:S42–9. [PubMed: 15596736]

29. Putnam WS, Prabhu S, Zheng Y, et al. Pharmacokinetic, pharmacodynamic and immunogenicity comparability assessment strategies for monoclonal antibodies. *Trends Biotechnol.* 2010 Aug 4. [Epub ahead of print].
30. Iznaga-Escobar N, Mishra AK, Perez-Rodriguez R. Factors affecting pharmacokinetics of monoclonal antibodies: A review article. *Methods Find Exp Clin Pharmacol.* 2004; 26:123–7. [PubMed: 15071611]
31. Powell DJ Jr, Felipe-Silva A, Merino MJ, et al. Administration of a CD25-directed immunotoxin, LMB-2, to patients with metastatic melanoma induces a selective partial reduction in regulatory T cells in vivo. *J Immunol.* 2007; 179:4919–28. [PubMed: 17878392]
32. Hassan R, Bullock S, Premkumar A, et al. Phase I study of SS1P, a recombinant anti-mesothelin immunotoxin given as a bolus I.V. infusion to patients with mesothelin-expressing mesothelioma, ovarian and pancreatic cancers. *Clin Cancer Res.* 2007; 13:5144–9. [PubMed: 17785569]
33. Garland L, Gitlitz B, Ebbinghaus S, Pan H, et al. Phase I trial of intravenous IL-4 pseudomonas exotoxin protein (NBI-3001) in patients with advanced solid tumors that express the IL-4 receptor. *J Immunother.* 2005; 28:376–81. [PubMed: 16000956]
34. Traini R, Kreitman RJ. Renal excretion of recombinant immunotoxins containing pseudomonas exotoxin. *Bioconjug Chem.* 2011; 22:736–40. [PubMed: 21410247]
35. Pai-Scherf LH, Villa J, Pearson D, et al. Hepatotoxicity in cancer patients receiving erb-38, a recombinant immunotoxin that targets the erbB2 receptor. *Clin Cancer Res.* 1999 Sep; 5(9):2311–5. [PubMed: 10499598]
36. Harandi A, Zaidi AS, Stocker AM, et al. Clinical Efficacy and Toxicity of Anti-EGFR Therapy in Common Cancers. *J Oncol.* 2009; 2009:567486. Epub 2009 May 6. [PubMed: 19424511]
37. Oh S, Stish BJ, Vickers SM, Buchbaum DJ, et al. A new drug delivery method of bispecific ligand-directed toxins, which reduces toxicity and promotes efficacy in a model of orthotopic pancreatic cancer. *Pancreas.* 2010; 29:913–22. [PubMed: 20182395]
38. Carriere D, Arcier JM, Derocq JM, et al. Antigenic modulation induced by four monoclonal antibodies adsorbed on gold particles (specificity anti-CD4, anti-CD5, anti-CD7, and anti-150-kDa antigen): relationship between modulation and cytotoxic activity of immunotoxins. *Exp Cell Res.* 1989; 182:114–28. [PubMed: 2785456]

Figure 1.

Construction of deimmunized EGF4KDEL. The gene fragment encoding the single-chain BLT dEGF4KDEL was created using overlap extension PCR. This construct consisted of an Nco1 restriction site, an ATG initiation codon, the genes for human EGF and circularly permuted human IL-4 linked by a 20 amino-acid segment of human muscle aldolase (HMA), the 7 amino-acid EASGGPE linker and the first 362 amino acids of the PE molecule with c-terminal KDEL.

Figure 2.

Anti-proliferative activity of EGF4KDEL. The *in vitro* activity of bispecific EGF4KDEL, monospecific EGFKDEL, monospecific IL4KDEL, and an equimolar combination of each monospecific drug was determined by measuring ³H-thymidine incorporation into MIA PaCa-2 cells following 72 hours incubation with varying concentrations of drug. Points represent mean of triplicate measures and standard deviation. Data is expressed as percentage of ³H-thymidine relative to control cells incubated in media alone (Control counts = $83,974 \pm 2,669$ cpm/20,000 cells). IC_{50} indicates the concentration of drug that inhibits 50% of cell proliferation relative to untreated cells.

Block EGF4KDEL with EGF4

Figure 3.

Blocking of EGF4KDEL drug activity. A blocking assay was performed to study the effect of EGF4, devoid of toxin on EGF4KDEL. MIA PaCa-2 cells were incubated with 1nM of EGF4KDEL and the effect of blocking with a 1, 10, 100, or 1,000 nM concentration of recombinant EGF4 was determined by measuring ^3H - thymidine incorporation. (Control counts= $100,725 \pm 6,490$ cpm/20,000 cells).

Figure 4.

The effect of dEGF4KDEL on additional EGFR⁺IL-4R⁺ pancreatic carcinoma lines. dEGF4KDEL and EGF4KDEL were tested against the pancreatic cancer cell lines SW1990 (A) and S2VP10 (B) using a ³H-leucine incorporation protein synthesis assay. CD3CD3KDEL was included as negative control. Data is expressed as percentage of ³H-leucine incorporation relative to control cells incubated in media alone.

Figure 5.

The effect of dEGF4KDEL on tumor progression in mice with MIA PaCa-2 orthotopic tumors. All nude mice were given an orthotopic injection of 4 million MIA PaCa-2 cells. Mice were treated with 3–4 μg of dEGF4KDEL IP. In Experiment 1, a single course of treatment of dEGF4KDEL consisted of an injection of drug given every other day (MWF). Treatment began on day 5 and mice were given 9 weekly courses of 4 μg /injection. In Experiment 2, a single course of treatment consisted of 2 injections of drug given every day (MTWTh). Treatment began on day 3 and mice were given 7 courses of 3 μg /injection. In Experiment 3, a single course of treatment consisted of 1 injection of drug given every day (MTWTh). Treatment began on day 5 and mice were given 7 weekly courses of 4 μg /injection. Animals were imaged weekly. Bioluminescence intensity was measured as a function of photons/sec/cm²/Sr. Histology of representative mice are also shown in the figure to confirm cancer free status of organs.

Average Tumor Growth

Figure 6.

Average tumor growth of mice in Figure 5, Experiment 1. Average total bioluminescent activity of mice treated with deimmunized EGF4KDEL drug (n=5/group), deimmunized negative control drug (d2219ARLKDEL), or no treatment (n=6/group) is shown.

Figure 7.

Anti-toxin levels of mice immunized with EGF4KDEL or dEGF4KDEL. Mice were immunized weekly with 0.25ug of EGF4KDEL (n=5) or dEGF4KDEL (n=5). Serum samples were collected 5 days after each immunization and IgG anti-toxin levels were determined by ELISA.