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Short Communication

Fusion Protein of Interleukin 4 and Diphtherial Toxin with High Cytotoxicity to Cancer Cells

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Abstract Receptor of human interleukin 4 (hIL4R) has been found to be present on many types of cancer, so it may be a good target for cancer therapy. Here, fusion toxin gene *DT4H* has been constructed by fusing DNA sequence encoding the first 389 amino acids of diphtherial toxin (DT), which can not bind its own receptor, to human interleukin 4 (hIL4) gene. In order to improve the affinity of fusion toxin for hIL4R, a circularly permuted form of hIL4 (cpIL4) was used. The fusion gene was expressed in *Escherichia coli* where the fusion toxin DT4H was highly expressed. Purified DT4H was very cytotoxic to cancer cell line U251 cells, and moderate cytotoxic to HepG2 and MCF-7 cells. SGC-7901 cells were insensitive to it. The cytotoxic action of DT4H was specific because it was blocked by excess hIL4. These results suggest that DT4H may be a useful agent in the treatment of certain malignancies.

Key words fusion toxin; diphtherial toxin; cancer therapy; interleukin 4; IL-4 receptor

One means to improve the selectivity of cancer therapy is by directing foreign protein with activity against therapeutic targets that displays different expression level on malignant cells from normal cells. There have had substantial efforts to rationally design these types of therapeutics such as targeted toxin [1].

A targeted toxin consists of a targeting polypeptide covalently linked to a peptide toxin. The targeting polypeptide (or ligand) directs the molecule to a cell surface receptor or determinant; the toxin moiety then enters the cell and induces apoptosis by, in most cases, inactivating protein synthesis. In this work, human interleukin 4 (IL-4) was employed as targeting ligand, since receptors for IL-4 have been found to be present on many types of cancers, such as breast cancers, lung cancers, melanomas, renal cell cancers, brain cancers and pancreatic cancers [2–7]. Diphtheria toxin (DT) is chosen for our studies because it is a toxin powerful enough to kill a cell with one molecule of DT enzymatic domain in the cytoplasm [8] and it has been successfully used in many targeted toxins. Williams *et al.* [9] described a series of internal in-frame deletion mutations that established 389 as the optimum site for genetic fusion of DT and targeting ligands.

In previous study [10], IL-4 based diphtherial fusion toxin has shown promising anti-tumor activity. But as some studies have indicated, the carboxyl terminus of IL-4 is important for binding [11,12]. The IL-4 fusion protein binds the IL-4 receptor with only 1% of the affinity of native IL-4 [13]. Kreitman *et al.* [14] developed a novel strategy for fusing two proteins in which the toxin was fused to the new C-terminus of circularly permuted form of IL-4 (cpIL4) and had showed good results. A circularly permuted protein is a mutant protein in which the native termini have been fused each other and new termini are created elsewhere in the molecule according to its three-dimensional structure.

Here, we fused DT389 to the N-terminus of cpIL4 with a 6×His tag in its C-terminus and evaluated its cytotoxicity in several cancer cell lines.

Materials and Methods

Materials

RNeasy minikit was from Qiagen; ThermoScript RT-

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PCR system was provided by Invitrogen; DNA marker, restriction endonucleases, and T4 DNA ligase were purchased from TaKaRa; the cloning and expression vectors, *E. coli* host strains were stored in our laboratory. All other chemicals were of analytical grade. The primers were synthesized by Sangon Inc., Shanghai.

Cell lines and cell culture

U251, derived from human glioma, was obtained from Institute of Biochemistry and Cell Biology (Shanghai, China) and maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Gibco). HepG2, MCF-7 and SGC-7901, originating from human liver, breast and stomach cancer respectively, were stocked in our lab, and maintained in DMEM medium (Gibco) with 10% FBS. All the cells were maintained at 37 °C in a humidified incubator of 5% CO_2.

Molecular cloning of mature human IL-4 and diphtheria toxin (DT) coding sequence

Coding sequence of IL-4 was obtained by RT-PCR from human white cells. Briefly, 200 μl whole blood sample was added in 3 ml fresh ammonium chloride lysing solution (150 mM NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM disodium EDTA, pH 7.4) and incubated for 10 min at room temperature. After centrifugation and wash with 2 ml PBS twice, the resuspended cells were conferred to total RNA extraction. Total RNA was extracted according to manufacturer's manual. About 1 μg of total RNA was reversely transcribed to cDNA using RT-PCR kit. After that, 2 μl of cDNA product was subjected to PCR to obtain the coding region of human IL-4 eliminating a signal peptide with sense primer (5′-aaa tcg aca cct att aat ggg tct cac-3′) and antisense primer (5′-tca gct cga aca ctt tga ata ttt ctc t-3′). The PCR amplification mixture (50 μl) contained 2 μl cDNA, 5 μl 10×PCR buffer, 200 μM dNTP, 1 u pyrobest polymerase and 0.5 μM of each primer. Amplification was performed under the following conditions: samples were subjected to 40 cycles of denaturation at 94 °C for 0.5 min, annealing at 54 °C for 0.5 min, extension at 72 °C for 1 min, and a final cycle of elongation for 10 min at 72 °C. The purified PCR product was ligated into pMD-18T vector, creating pIL4-T. The construction was confirmed by restriction mapping and sequencing.

Coding sequence of diphtheria toxin lacking of signal peptide came from PCR amplification of genome DNA of *Corynebacterium diphtheriae* (CMCC 38003). Extraction of genome DNA was operated according to *Molecular Cloning: A Laboratory Manual* (2nd ed). DT was PCR amplified with primer 5′-ggc gct gat gat gtt gtt g-3′ as the forward and 5′-ggc aca cga ccc cac tac ctt t-3′ as the reverse. The PCR mixture was similar to that of IL-4, and the condition was at 94 °C 4 min, followed by 30 cycles of 94 °C 30 s, 54 °C 30 s and 72 °C 3 min, and finally 72 °C for 7 min. The final product was also ligated into pMD-18T, creating pDT-T, for sequencing.

Constructions of the plasmid expressing fusion protein

The sequences of oligonucleotide primers used in these constructions are as follows:

(a) 5′-**ggc ggt aac ggt ggc** cac aag tgc gat atc acc tta ca-3′ (GGNGG codons in bold); (b) 5′-gc**a agc ttc** ttg gag gca aag atg tc-3′ (*Hin*dIII site in bold); (c) 5′ gct **gga tcc** atg aac aca act gag aag gaa acc t-3′ (*Bam*HI site in bold); (d) 5′-**gcc acc gtt acc gcc** gct cga aca ctt tga ata ttt ctc-3′ (GGNGG codons in bold); (e) 5′-tcg **cat atg** ggc gct gat gat gtt gtt ga-3′ (*Nde*I site in bold); (f) 5′-ttc **gga tcc** acc aga agc ctc aaa tgg ttg cgt ttt atg ccc cg-3′ (*Bam*HI site in bold).

DT389, encoding a truncated diphtheria toxin which contains amino acid 1–389 of DT with an additional Met in the N-terminus, was generated by PCR amplification of pDT-T with primer (e) as the sense and (f) as the antisense. Then DT389 fragment digested with *Nde*I and *Bam*HI was ligated into the *Nde*I and *Bam*HI sites in the pT7473 plasmid (derived from pET21a), creating pDT389.

pDT4H encodes a 6×His fusion protein of DT389 and cpIL4. cpIL4 is a circularly permuted human IL-4, which consists of 38–129 aa and 1–37 aa of native IL-4 connected by linker GGNGG. So pDT4H contains "atg", 1–389 aa codons of DT, gag-gct-tct-ggt-gga-tcc-gaa between the toxin sequence and IL-4 codon 38, IL-4 codons 38–129, ggc-ggt-aac-ggt-ggc encoding the GGNGG linker, IL-4 codons 1–37, and 6×His tag codons. DNA encoding cpIL4 was constructed by the method of gene splicing by overlap extension (SOE) as described [15]. Briefly the first part (1–37 aa codons) and the second part (38–129 aa codons) of mature IL-4 mRNA were amplified separately using primers (a, b) and (c, d), so that the 5′ end of the first fragment and the 3′ end of the second fragment were identical and encoded the GGNGG linker. The two fragments therefore annealed in reverse order to form a template for the final PCR step using primers b and c. Primers (c, b) created *Bam*HI and *Hin*dIII restriction sites and the *Bam*HI-*Hin*dIII fragment of the final product was ligated to the sites in pDT389, to get pDT4H.

Protein expression and purification

Overnight cultures of *E. coli* BL21(DE3) transformed with pDT4H were stored as glycerol stocks at –80 °C, streaked for confluency on LB-agar plates containing 0.1 g/L of ampicillin and cultured at 37 °C. Bacteria, from a single plate, were collected, inoculated into 1 L super broth containing 5% glucose, 1.6 mM $MgSO_4$, 0.1 g/L ampicillin, and shaken at 37 °C at 250 r/min, to an optical absorbance of 2.0 at 650 nm, then 1 mM isopropylthio-β-*D*-galactoside (IPTG) was added and cells were cultured for an additional 90 min. Cultures were centrifuged at 4000 *g* for 10 min. The cells were suspended in 50 mM Tris-HCl (pH 8.0) for 1 h and added Triton X-100 at the final concentration of 0.25% and sonicated. The suspended buffer was centrifuged and the pellet was washed by the same treatment. The pellet inclusion body was precipitated and solubilized by 100 mM Tris-HCl (pH 8.0) containing 8 M urea to a protein concentration of 10 mg/ml. Dithioerythritol was added to 65 mM, and after overnight incubation at 22 °C the reduced denatured protein was diluted 100-fold into 100 mM Tris-HCl, pH 8.0, 0.5 M *L*-arginine, 0.9 mM oxidized glutathione, 2 mM EDTA. After incubation at 10 °C for 36–48 h, the refolded protein was dialyzed into 20 mM Tris, 100 mM urea. The dialyzed protein was loaded on DEAE-Sepharose Fast Flow and eluted by 0.3 M NaCl. After dialyzed against 20 mM Tris, 100 mM urea (pH 7.4), the protein solution was loaded on Ni^{2+}/NTA according to the manufacturer's protocol. Briefly, samples were loaded onto an equilibrated 4 ml Ni^{2+}/NTA affinity matrix, the column was washed with 20 ml binding buffer, then eluted contaminating proteins from the resin with 10 ml low concentrations of imidazole (0.075 M). The recombinant proteins were eluted with the addition of binding buffer containing 0.25 M imidazole. The purified product was dialyzed against PBS.

Cytotoxicity assay

After exposure to drugs, the viability was determined by the MTT assay following published procedures [16]. Briefly, 10,000–30,000 cells in 200 μl of medium were plated onto each well of 96-well tissue culture plate. After overnight of culture, various concentrations of fusion toxin and native IL-4 (R&D) were added to the medium. Cells were cultured for 36 h. After exposure, the medium was aspirated and replaced with 200 μl of fresh drug-free medium containing 0.5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide]. The cells were incubated for an additional 4 h. This medium containing MTT was removed by inverting the plate and 200 μl of 15% SDS, 15 mM HCl were then added to each well. After 30 minutes of incubation at 37 °C, absorbance in each well was read in a microplate reader at 450 nm. All experiments included untreated control cells, they were repeated three times and each concentration was tested in triplicate. The result was expressed as a percent of the control without any toxin.

Clonogenic assay

The *in vitro* cytotoxic activities of IL-4 cytotoxin on U251 cells were also determined by colony-forming assay. The cells were plated in triplicate in 100-mm Petri dishes with 7 ml of medium containing 10% fetal bovine serum and allowed to attach for 20–22 h. The number of cells/plate was chosen so that >100 colonies were obtained in the control group.The cells were exposed to different concentrations of IL-4 cytotoxin (0–100 ng/ml) for 9 d at 37 °C in a humidified incubator. The cells were washed, fixed, and stained with crystal violet (0.25% in 25% ethanol). Colonies consisting of >50 cells were scored. The percentage of colony survival was determined from the number of colonies remaining in the treated groups divided by the number of control group colonies.

Results

Cloning of the coding sequence of human IL-4 and diphtherial toxin and construction of fusion toxin

IL-4 was obtained by RT-PCR from human blood white cells, and sequence was identical to M13982 (GenBank). The construction of DT4H is shown in Fig. 1. Diphtherial toxin comes from PCR amplification of genome DNA of *Corynebacterium diphtheriae.* Its sequence was identical

Fig. 1 Schematic drawing of DT4H

DT4H was constructed by fusing DT389 to cpIL4 with a spacer sequence. cpIL4 consists of 38–129 aa and 1–37 aa of native IL-4 connected by a linker. A 6×His tag was retained at the C-terminal. Spacer: EASGGSE; linker: GGNGG.

to K01722 (GenBank). In order to construct a fusion toxin efficiently killing cancer cells, the truncated form of DT, which is missing the native cell-binding domain, was employed. And it was linked to a circularly permuted IL-4 by a spacer sequence EASGGSE. 6×His tag was fused to C-terminus further for convenient purification.

Protein expression and purification

The protein encoded by plasmid pDT4H was expressed in *E. coli* BL21(DE3) under the control of a bacteriophage T_7 late promoter. The fusion protein represented the main band on SDS-PAGE analysis of the total cell extract and was exclusively present in the form of inclusion bodies. The inclusion bodies were dissolved in 8 M urea, and the protein, already in highly purified form, was subjected to renaturation and purification as previously described in Method. DT4H appeared as a single entity and eluted in fractions expected for a protein with a molecular mass of 60 kD demonstrating 90% purity of the final product (Fig. 2,3).

Cytotoxicity of DT4H to several human cancer cell lines

We tested several cell lines to determine if DT4H is cytotoxic to them. We examined cancers derived from brain, breast, stomach and liver. A glioma cell line, U251, was very responsive to DT4H with an IC_{50} of 4 ng/ml. MCF-7 breast cancer and liver cancer HepG2 cell lines had poorer responses with IC_{50} of 300 ng/ml and 200 ng/ml, respectively. The gastric SGC-7901 was insensitive to the toxin (data not shown).

The specificity of DT4H cytotoxic action was tested

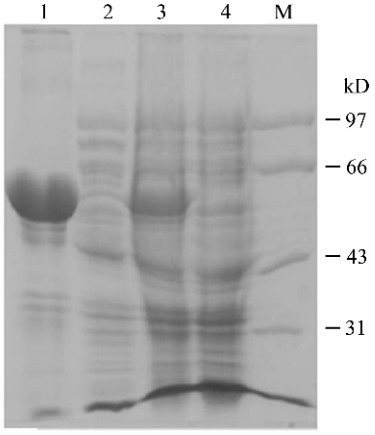

Fig. 2 SDS-PAGE analysis of expression of DT4H
M, marker; 1 and 2, inclusion bodies and supernatant after sonication; 3 and 4, lysed *E. coli* after and prior to IPTG induction, respectively.

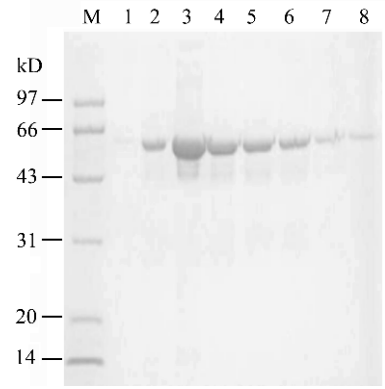

Fig.3 Analysis of DT4H purification with SDS-PAGE
M, marker; 1–8, sequential fractions eluted from Ni^{2+}/NTA column by 0.25 M imidazole.

with block effect of excess recombinant human IL-4 (rhIL4). rhIL4 showed the inhibition of cytotoxic action of DT4H at a concentration of 0.25 μg/ml. This may be caused by the lack of IL-4 receptor on the surface of SGC-7901 cell (Fig. 4).

Inhibition of colony formation of U251 cell line by IL-4 cytotoxin

We performed a colony-formation assay using the U251 cell line. Two hundred cells were plated in each dish and incubated with various concentrations of IL-4 cytotoxin. After 9–14 days of culture, the percentages of colonies formed in control and IL-4 cytotoxin-incubated groups were compared. IL-4 cytotoxin inhibited colony formation in U251 cell line in a concentration-dependent manner. The number of colonies in untreated groups served as the 100% control value. Less than 10 ng/ml of IL-4 cytotoxin inhibited colony formation by 50% in U251 cell line. These results were comparable with the dose-dependent kinetics observed in the cytotoxicity assay (Fig. 5).

Discussion

IL-4 receptor (IL-4R) exists in three different types—type I, II and III [17,18]. Current studies show that IL-4 R is specifically expressed on the surface of many tumor cells, especially type II [18]. IL-4Rα chain by itself can mediate internalization of receptor complex [19], which is a critical process for cytotoxicity action of IL-4 toxin.

It is known that normal resting B cells, T cells, monocytes, and resting or activated bone marrow precursor (CD34) cells which express low level of IL-4 receptors

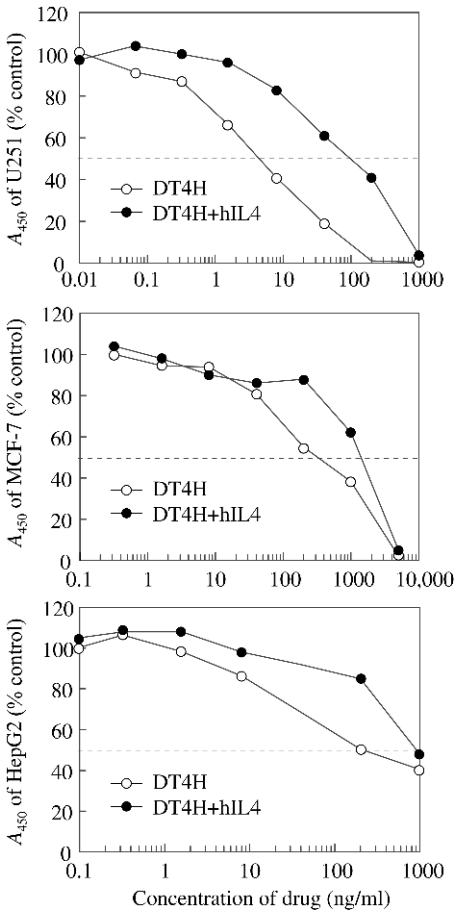

Fig. 4 Cytotoxic activity of DT4H on several cancer cell lines and the inhibition of this cytotoxicity by hIL4

The result was expressed as a percent of the control (without any toxin). hIL4 was added at a concentration of 0.25 μg/ml. The dashed line shows 50% of viability.

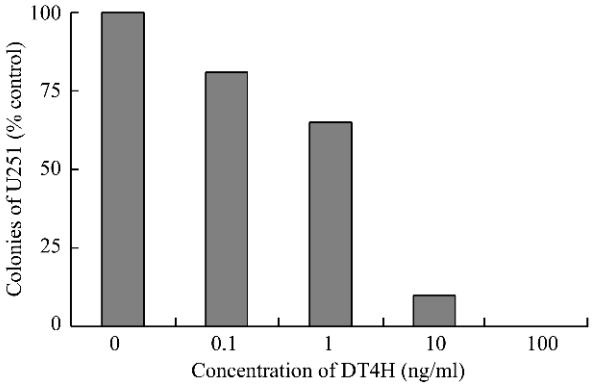

Fig. 5 Inhibition of colony formation by IL-4 cytotoxin

Two hundred U251 cells were allowed to adhere in Petri dishes and cultured with various concentrations (0–100 ng/ml) of DT4H for 9–14 days, the percentages of colonies formed in control and IL-4 cytotoxin-incubated groups were compared.

are not sensitive to cpIL4-PE [20,21]. Also, normal endothelial and fibroblast cells, brain, skin, and diseased kidney tissue samples seem to express IL-4R, but it is not clear what extent this chain was expressed. So the toxicological significance of IL-4 toxin on these tissues is difficult to predict. However, the toxicological experiments of cynomolgous monkeys with systemic administration of IL4 (38–37)-PE38KDEL did not show any toxicities in other vital organs in these monkeys except for an elevation of hepatic transaminases, which is a general side effect observed in treatments with many fusion toxins but not limited to IL-4 toxin [1]. So, it may not be caused by IL-4R. These results suggest that there is a large therapeutic window in which one can target IL-4 toxin to cancer cells but cause little collateral damage to immune and non-immune tissues.

Since the C-terminus of native hIL-4 participated in the interaction with its receptor, former fusion toxin IL-4 and *Pseudomonas* exotoxin A showed low affinity. Then circularly permuted IL-4 was constructed based on its spatial structure. Another point which should be noted is that Asn^{38} of IL-4 has been found to be glycosylated in eukaryotic cells and deglycosylated form of the human IL-4 retains the same function [22,23]. So functional *N*-glycosylation sites should be good candidate locations for circular permutation of some molecules.

There has been a long history of constructing targeted toxins for human cancer treatments. The toxins most commonly modified for the construction of targeted molecules that have been clinically evaluated including DT and PE from bacteria, and ricin, Gel and PAP isolated from plants [1]. Currently, there is insufficient information to choose one toxin over another in the assembly of a targeted toxin for a particular malignancy. However, ONTAK, composed of DT389 fused to human IL-2, has received Food and Drug Administration approval for sale in 1999 [24]. So there appears a reasonable preference of employing DT for construction.

In conclusion, we have fused 1–389 aa of diphtheria toxin (DT389) to cpIL4 and made a fusion toxin, DT4H, with a 6×His tag in its C-terminus. It can be expressed in BL21(DE3) and obtained by simple two steps of purification with >90% homogeneous and typical yield of 5–8 mg/L culture. Its cytotoxicity was determined in several cell lines. Among them, U251 derived from human glioma was very sensitive. The reason may be due to the high expression level of IL-4 receptor on the cell surface. Since clonogenicity *in vitro* correlated with *in vivo* malignant phenotype in xenografts, DT4H inhibition on colony formation was tested in U251. The result was consistent with that of cytotoxicity assay. These results show DT4H may be a potential agent for cancer therapy.

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