Novel EGFR-bispecific recombinant immunotoxin based on cucurmosin shows potent anti-tumor efficiency in vitro

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Abstract. Epidermal growth factor receptor (EGFR) is overexpressed in various tumors and is associated with cancer initiation, progression, and poor prognosis. Despite the achievements made by tyrosine kinase inhibitors and monoclonal antibodies in certain cases, many patients have not benefited from such treatment due to resistance. Immunotoxins (ITs) are antibody-cytotoxin chimeric molecules with specific cell killing ability, which have achieved different degrees of success in the treatment of a wide range of cancers in clinical trials. The aim of the current study was to examine a novel targeting EGFR recombinant immunotoxin Bs/cucurmosin (CUS) generated by fusing CUS to the EGFR-specific nanobody 7D12-9G8. Bs/CUS was successfully expressed in Escherichia coli strain BL21 (DE3) in a soluble form. Furthermore, it retained binding capacity and specificity with EGFR and was superior to rE/CUS, a monospecific IT we reported previously. In vitro results showed that Bs/CUS could be internalized into the cytoplasm and selectively kill cells in the picomolar range. Flow cytometry showed that Bs/CUS killed the cells mediated by the apoptosis pathway. Taken together, results of the current study indicated that Bs/CUS is a promising candidate that should be further evaluated as a cancer therapeutic for the treatment of EGFR-positive tumors.

Introduction

The epidermal growth factor receptor (EGFR) family is a transmembrane protein receptor with tyrosine kinase activity (1,2). EGFR is overexpressed in more than 60% of triple-negative breast cancers (TNBCs), as well as in non-small cell lung cancers (NSCLC), colorectal cancer (CRC), and glioblastoma (3,4). It can activate several downstream signaling pathways related to cancer when the EGFR extracellular domain binds to its ligands. Given the important role of EGFR in multiple cancer processes such as proliferation and metastasis, various tyrosine kinase inhibitors (TKIs) and monoclonal antibodies (mAbs) have been developed for targeting EGFR in human cancers. Although EGFR-targeted therapies have shown success in certain cases, many patients fail to respond because tumors inevitably develop acquired resistance (5-7). Therefore, it is crucial to identify other treatments in the prevention of cancer.

One of the therapeutic methods that can meet this need is immunotoxins (ITs). ITs are antibody-cytotoxin chimeric molecules with specific cell killing ability (8). To date, unprecedented progress in treating hematological tumors has been achieved. Lumoxiti (moxetumomab pasudotox-tdfk) is a first-class anti-CD22 recombinant IT that fuses the binding domain of the anti-CD22 antibody to PE38 (9). It has been approved by the FDA as an intravenous fluid for the treatment of adult patients with recurrent or refractory hairy cell leukemia (HCL) (9). However, ITs are not as optimistic as hematologic malignancies in the treatment of solid tumors (10,11). The capacity of IT to penetrate solid tumors (12), the generation of antitoxin antibodies (9,13), and their clearance through kidney filtration (14) all limit the role of IT therapy in the treatment of solid tumors.

Nanoantibody, also known as single-domain antibody (sdAb), is the smallest known antigen-binding fragment available, preserving the full binding capacity of an intact antibody (15). Compared with conventional antibodies, the benefits of nanobodies include ease of expression, low molecular weights, good tissue penetration, high stability and solubility, and refolding ability; these benefits have granted their applications for the medical and biotechnological fields (15).

A novel typical type I ribosome-inactivating protein (RIP) known as cucurmosin (CUS) was isolated from the sarcocarp of Cucurbita moschata by our group with a determined DNA sequence and spatial structure (16). CUS can inhibit the growth

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of different human tumor cells in vitro and in vivo, including, but not limited to, PANC-1 (17), HepG2 (18), CFPAC-1 (19), and SW-1990 (20), but it presents low toxicity to normal cells. Compared with other RIPS, such as luffaculin and trichosanthin, the cytotoxicity of CUS is 4– to 7-fold stronger. These data indicated that CUS can be used as a toxic component of ITs targeted at tumor cells.

We previously reported an anti-EGFR nanobody 7D12-based recombinant immunotoxin rE/CUS (21), which can selectively kill EGFR cells in vitro. 9G8 is another anti-EGFR nanobody with different epitope specificities compared with 7D12 (22). Previous findings showed that 7D12 and 9G8 did not compete for binding to EGFR (22). To improve the binding ability of rE/CUS and creation of more potential ITs, the bispecific nanobody 7D12-9G8 was used instead of 7D12, which is capable of binding EGFR, and conjugated CUS using a flexible linker (G4S), by genetic engineering methods. The bispecific recombinant IT known as Bs/CUS is more sensitive to cancer cell lines with EGFR expression and has a stronger cytotoxic effect than rE/CUS. The selectivity and cytotoxicity of Bs/CUS illustrate its potential as a novel candidate for treating EGFR-overexpressing tumors (22).

The aim of the current study was to examine a novel targeting EGFR recombinant immunotoxin CUS generated by fusing CUS to the EGFR-specific nanobody 7D12-9G8. The results indicated that Bs/CUS is a promising candidate that should be further evaluated as a cancer therapeutic for the treatment of EGFR-positive tumors.

Materials and methods

Reagents. Cetuximab was obtained from Merck. Goat pAb to human IgG (FITC) (ab81051; Abcam), Rb pAb to 6X His-tag® (FITC) (ab1206; Abcam), mouse-IgGK BP-HRP (sc-516102; Santa Cruz Biotechnology), and mouse anti-6X His Tag® antibody (ab18184; Abcam) were used in the present study. Goat-anti-mouse IgG-APC (550826; BD Bioscience), and FITC conjugate goat anti-mouse IgG (AMS.AS1105-1000; Boster Biological Technology) were also used. Mouse anti-CUS antibody, IG9, was produced by our laboratory. Escherichia coli strain BL21 (DE3), plasmid pET32a (Sangon Biotech), and Ni-NTA Sepharose FF (GE Healthcare) were utilized.

Cell lines. Human hepatoma cell line HepG2, human NSCLC cell line A549, and human CRC cell lines SW1116 and SW620 were obtained from the cell bank/stem cell bank of the Chinese Academy of Sciences. All cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). All the cell lines used were authenticated by short tandem repeat-based DNA profiling.

Production and characterization of recombinant IT. Anti-EGFR bispecific nanobody 7D12-9G8 was cloned into pET32a expression plasmid-containing CUS (designed Bs/CUS). The expression and purification of Bs/CUS were performed following previously described protocol (21).

Western blot analysis and 12% SDS-PAGE gel were used to analyze the purified protein. The mouse-anti-His tag antibody and mouse anti-CUS antibody were used as primary antibodies, and mouse-IgGk BP-HRP was used as the secondary antibody.

EGFR expression and Bs/CUS binding capability. Cell surface EGFR expression of A549, HepG2, SW1116, and SW620 cells and binding ability of Bs/CUS were assessed by flow cytometry. To evaluate cell surface EGFR expression, cells were incubated with cetuximab and detected by anti-human-IgG (FITC). To verify Bs/CUS binding ability, 1 µg of rE/CUS and Bs/CUS was incubated with the abovementioned cell lines at 4°C for 30 min, respectively. The mouse-anti-6X His tag antibody was added, followed by the detection antibody anti-mouse-IgG (APC), and analyzed by flow cytometry.

In vitro cytotoxicity assay. Sulforhodamine B (SRB) was applied to assess the cytotoxic activity of drugs as previously described (21). In brief, cells (3x10^4/ml per well) were added into 96-well plates in RPMI-1640 medium and incubated overnight at 37°C in 5% CO2. CUS, 7D12-9G8, rE/CUS, Bs/CUS, and CUS+7D12-9G8 at different concentrations were added for 72 and 120 h, respectively. Absorbance was measured by using an Epoch Microplate Reader (BioTek instrument, Inc.) at 515 nm. Cytotoxic activity was defined by IC50 values.

Apoptosis assay. The FITC-Annexin V apoptosis kit (KeyGen Biotechnoloe Co., Ltd.) was used to evaluate the apoptotic effects of Bs/CUS on HepG2 cells. Cells at a density of 4x10^4/ml per well were seeded in 6-well plates, incubated overnight at 37°C and treated for 72 h with Bs/CUS or kept untreated (UT) at concentrations of 1, 4, 16, and 64 nmol/l. Subsequently, the cells were collected, centrifuged at 500 x g for 5 min at room temperature, counted, 5 µl Annexin V-FITC and 5 µl PI were added for 15 min at room temperature in the dark for staining, resuspended in 500 µl binding buffer, and analyzed by flow cytometry (BD Biosciences).

Cell cycle analysis. Cell cycle changes induced by Bs/CUS treatment were studied by flow cytometry. In preparation for the cell cycle assay, HepG2 cells (1x10^6 cells/well) were seeded in 6-well plates and incubated overnight at 37°C. The cells were then treated with Bs/CUS or kept untreated (UT) at concentrations of 1 and 4 nmol/l and incubated with serum-free medium for 24 and 48 h, respectively. Cells were collected by EDTA-free trypsinization, fixed in 70% ethanol, and treated with PI solution (50 µg/ml PI and 100 µg/ml RNase A) (Sigma-Aldrich; Merck KGaA) for 30 min at 37°C. Flow cytometry was used to analyze the cell cycle status of treated HepG2 cells.

Confocal microscopy. HepG2 cells were seeded in glass bottom cell culture dishes at a density of 1x10^5 cells per well and allowed to attach overnight. The next day, the cells were incubated with 5 µg/ml Bs/CUS for 3, 6, and 9 h, respectively. The cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature and 0.5% Triton X-100 permeate for 15 min. The cells were then washed with cold PBS and incubated in blocking buffer (PBS containing 5% bovine serum albumin) at room temperature for 30 min, followed by incubation with the primary antibody IG9 at 4°C overnight. Cells were washed with cold PBS three times and incubated
with the indicated goat anti-mouse IgG (FITC) secondary antibody for 1 h at room temperature. DAPI was applied to stain the cell nuclei at room temperature for 15 min. Images were obtained by confocal microscopy using an LSM 710 system (Carl Zeiss) with 63x water C-Apochromat objective.

**Statistical analysis.** Each experiment was repeated in triplicate to determine the reproducibility of the results. Experimental data were presented as mean ± SD. A one-way ANOVA followed by Dunnett’s multiple comparison test and Student’s t-test were used to analyze the statistical comparisons. P<0.05 was considered statistically significant.

**Results**

**Generation and characterization of Bs/CUS.** The recombinant plasmid of pET32a(+)/Bs/CUS was constructed as shown in Fig. 1A. Bs/CUS and CUS were expressed in *E. coli* BL21 (DE3) and analyzed using 12% SDS-PAGE gel (Fig. 1B). Western blot analysis was applied to confirm the recombinant proteins Bs/CUS and CUS by using the primary antibodies against CUS and His tag, respectively. 1, Bs/CUS; 2, CUS. (E) EGFR expression on different cell lines. (F and G) Affinity of Bs/CUS compared with that of rE/CUS by flow cytometry. MFI, mean fluorescence intensity. *P<0.05 vs. SW620.*
recombinant IT-Bs/CUS was successfully expressed in the *E. coli* prokaryotic expression system and purified.

The EGFR expression at the cell surface level was analyzed by flow cytometry using cetuximab as a primary antibody. As shown in Fig. 1E, A549 and HepG2 had the higher EGFR expression level (P<0.05), while SW1116 had relatively lower expression levels. SW620 cells were considered EGFR-negative cells.

Flow cytometry was applied to analyze the binding characteristic of Bs/CUS and compared with that of rE/CUS. Fig. 1F and G show that the fluorescence intensity of Bs/CUS was higher than that of rE/CUS, indicating that Bs/CUS had stronger binding activity than rE/CUS. The rank order of the sensitivities of the cell lines to Bs/CUS in the EGFR expression level was A549>HepG2>SW1116>SW620, which was consistent with cetuximab. These results demonstrated that Bs/CUS retained the binding capacity and specificity with EGFR and was superior to rE/CUS.

### Table I. IC50 values of cells treated with drugs for 72 and 120 h.

| Cell lines          | Bs/CUS (nmol/l) | rE/CUS (nmol/l) | CUS (nmol/l) | CUS+7D12-9G8 (nmol/l) | IT-1 | IT-2 |
|---------------------|----------------|----------------|--------------|-----------------------|------|------|
| A549 (72 h)         | 0.018±0.011     | 1.300±0.712     | 215±0.106    | 257±0.097             | 9760 | 102.3|
| A549 (120 h)        | 0.008±0.001     | 0.623±0.172     | 180±0.026    | 170±0.031             | 22500 | 288.9|
| HepG2 (72 h)        | 0.025±0.011     | 2.386±0.624     | 244±0.033    | 337±0.015             | 9760 | 102.3|
| HepG2 (120 h)       | 0.008±0.002     | 0.551±0.163     | 109±0.009    | 187±0.010             | 13625 | 197.8|
| SW1116 (72 h)       | 0.138±0.107     | 7.665±2.602     | 192±0.023    | 261±0.047             | 1391 | 25.05|
| SW1116 (120 h)      | 0.025±0.011     | 1.820±0.884     | 74±0.016     | 72±0.007              | 2960 | 40.66|
| SW620 (72 h)        | 186.8±41.96     | 494.2±73.79     | 550±0.201    | 471±0.089             | 2944 | 0.001|
| SW620 (120 h)       | 88.89±5.71      | 305±19.83       | 202±0.11     | 169±0.023             | 2.272 | 0.662|

*P<0.01 vs. CUS; *P<0.01 vs. Bs/CUS. TI-1: Target index-1=IC50^{CUS}/IC50^{Bs/CUS}; TI-2: Target index-2=IC50^{CUS}/IC50^{rE/CUS}.

Cytotoxicity in vitro. An SRB-based assay was applied to determine the specific cytotoxicity of Bs/CUS *in vitro*. The various tumor cell lines were incubated with decreasing concentrations of CUS, 7D12-9G8, rE/CUS, Bs/CUS, and CUS + 7D12-9G8 for 72 and 120 h. The proteins were found to be cytotoxic in a dose-dependent manner on target cells (Fig. 2A). rE/CUS and Bs/CUS were significantly more cytotoxic than CUS alone (P<0.05). For the 72-h experiments, the IC50 of Bs/CUS was >0.1 nmol/l but decreased to 0.02 nmol/l for SW1116, which indicated a 78-fold increase compared with that of rE/CUS. Thus, Bs/CUS and CUS + 7D12-9G8 did not increase in comparison with both CUS and rE/CUS.

Annexin V/PI assay was applied to determine whether the cytotoxicity of Bs/CUS is caused by apoptosis, and the data shown in Fig. 2B and C. The results indicated a significant increase in early and late apoptosis in Bs/CUS in comparison with the untreated control. The apoptosis rate increased with the increasing concentration of Bs/CUS. The percentage of apoptosis was 22.45±0.21, 69.60±0.14, 66.50±0.42 and 74.15±2.33, respectively, in Bs/CUS-treated HepG2 at different concentrations. When the concentration of Bs/CUS was half that of rE/CUS (8 nmol/l), the apoptosis rate was higher than that of rE/CUS. Thus, Bs/CUS was found to be highly efficient in inducing apoptosis-mediated cell death.

Cell cycle analysis. HepG2 cells were treated with different doses of Bs/CUS (PBS, 1 and 4 nmol/l) for 24 and 48 h, and flow cytometric analysis was carried out to detect the cell cycle distribution via PI staining. For the 24 h test, it was found that Bs/CUS caused arrest in the G0/G1 phase and the 48 h test compared with the control group (Fig. 3). The proportion of cells incubated with 4 nmol/l Bs/CUS significantly increased in the G0/G1 phase and decreased in the proportion of cells in the S and G2/M phases (10.58 and 4.81%, respectively; P<0.0001).

Internalization of Bs/CUS. Indirect immunofluorescence was applied to confirm the internalization of Bs/CUS in HepG2 cells. As shown in Fig. 4, the fluorescent signal was detected mainly on the cell surface treated with Bs/CUS for 3 h. For 6 h, signals started to accumulate in the cytoplasm, and the fluorescence signal was detected both in the cytoplasm and membrane. At incubation periods longer than 9 h, the internalized Bs/CUS protein appeared as evenly distributed dots in the cytoplasm, while the cell membrane showed no luminescence, indicating that it had completely entered the cytoplasm.
Discussion

EGFR is overexpressed in various tumors and is associated with cancer initiation, progression, and poor prognosis, by mutations, gene amplification, or both through constitutive EGFR activation (3,4). Cancer therapies targeting EGFR, such as TKIs and mAbs, have been developed as standard therapies for several cancers including but not limited to NSCLC and CRC (23-25). The main limitation of treatments targeting EGFR is the appearance of acquired resistance. Immunotoxins function not by suppressing receptor-mediated signaling but by directly killing the cells. In this study, CUS, the toxin moiety used is a typical type I RIP. It can hydrolyze the N-glycosidic bond at A4324 on the 28S rRNA of eukaryotic cells, irreversibly inactivating the ribosomal 60S subunit, which halts protein synthesis. Thus, there can be less chance for tumor cells to upregulate rescue mutations or alternative signaling pathways to resist immunotoxin therapy (26).

Given the importance of EGFR in solid tumors, we constructed a recombinant IT by using the anti-EGFR bispecific nanobody 7D12-9G8 fused to a toxin known as Bs/CUS and demonstrated the anti-tumor activity of the IT Bs/CUS in vitro. The conventional recombinant IT molecules are constructed by fusion of the toxin with scFv or dsFv, which often have the problems of stability, water solubility, and aggregation (27). Nevertheless, the IT Bs/CUS based on bispecific nanobody did not have the abovementioned problems, possibly due to the beneficial outcomes of the nanobody, so it could be directly expressed in the E. coli system as soluble proteins.

According to previous findings, when the various tumor cell lines were incubated with decreasing concentrations of CUS for 24, 48, 72, 96, and 120 h, the proteins were found to be cytotoxic in a time-dependent manner on target cells (17-19). Results of the cytotoxicity assay in vitro showed that the killing ability of Bs/CUS on different tumor cells was time-dependent under the conditions of 72 and 120 h incubation. This result and incubation time are consistent with the research results of other immunotoxins using CUS as a toxic agent in our laboratory (28,29).
On the basis of the combination tests of flow cytometry, SRB cytotoxicity, and apoptosis assay, our results showed that the IT Bs/CUS had higher EGFR binding capacity and binding specificity than rE/CUS. These results were consistent with previous reports showing that the bi-paratopic 9G8-7D12 molecule had a higher affinity than 7D12 and 9G8 alone, respectively, due to their different binding sites on the EGFR receptor (22). However, there was no statistical difference between the affinity of rE/CUS and Bs/CUS. The binding ability of Bs/CUS was lower than the chemically-linked conjugates, T-CUS245C, and D-CUS245C, which were constructed by our group. Therefore, future studies are necessary to understand the Kd value of Bs/CUS and further improve it. A comparison with other bispecific ITs also reflected the high potency against tumors of Bs/CUS, such as dDT2219, an RIT with an IC50 range of 0.23-1.03 nmol/l against B-cell malignancies (30).

Nevertheless, we have verified that Bs/CUS exerts its function of killing tumor cells through the apoptotic pathway by annexin V/phycocerythrin-based apoptosis assay. To make the results solid, more verification experiments such as CCK8, DraQ7, and immunofluorescent staining using apoptosis-related antibody should be utilized in subsequent investigations. In addition, in the present study, we preliminarily measured that Bs/CUS induced HepG2 cell arrest in the G0/G1 phase; however, the molecular mechanism of cell cycle arrest involved in Bs/CUS remains to be verified.

Given that the rE/CUS has a molecular weight of approximately 42 kDa, it could be cleared rapidly in vivo via the kidneys. Although Bs/CUS is an IT based on the fusion of bispecific nanobody with a molecular weight of 58 kDa, it may also face the above problem due to its size under the glomerular filtration threshold (70 kDa) (31). Accumulating evidence from multiple trials indicated that binding to albumin is an exceptional option to prolong the in vivo half-life of small proteins (14,32,33). Thus, future studies are necessary to verify the effectiveness of Bs/CUS with the albumin-binding domain in vivo.

Taken together, Bs/CUS demonstrates high cytotoxicity and selectivity on EGFR-positive cancer cells, indicating that it could be a promising candidate and it should be further evaluated as a cancer therapeutic for the treatment of EGFR-positive tumors.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CZ and YC contributed to study concept and design, acquisition of data, analysis and interpretation of data, and drafting of the manuscript; XD performed the experiments; JW conducted additional experiments; YL contributed to the statistical analysis; HZ, ML, and JL contributed to the study concept, study supervision and critical revision of the manuscript; JX contributed to the study concept and design, study supervision and critical revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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