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Xu Ming-Kai · Zhang Cheng-Gang

Gene expression and function study of fusion immunotoxin anti-Her-2-scFv—SEC2 in *Escherichia coli*

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Abstract The whole *sec2* DNA fragment was obtained by polymerase chain reaction with total genomic DNA extracted from *Staphylococcus aureus*. The newly discovered gene contains 717 bp (GenBank Accession number AY450554) and encodes 239 amino acids in accordance with the protein sequence reported in GenBank. The *sec2* gene was fused to anti-Her-2 scFv gene with a DNA linker at the upstream to construct the fusion gene of immunotoxin which was subcloned to expression vector pET-32a (+) and expressed efficiently in *Escherichia coli*. The purified fusion immunotoxin could target the HER-2 overexpressed by breast tumor cell SK-Br-3 in vitro and demonstrate tumor-inhibition effect on SK-Br-3.

Introduction

As superantigens, staphylococcal enterotoxins (SEs) possess the capacity of interacting with the Vβ regions of the T-cell receptor and major histocompatibility complex (MHC) II (Balaban and Rasooly 2000). Through this interaction, SEs activate a large number of T cells to release massive cytokines, including interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF), which launch a powerful effect of tumor inhibition in vivo as well as in vitro (Dohlsten et al. 1993; Pardoll 1995). As a special member of the SE family, SEC has three highly conservative subtypes: SEC1, SEC2, and SEC3. In China, SEC2 has been used in clinics as an effective therapeutic agent for tumor treatment, and some encouraging results have been reported (Chen 2001). However, matured tumor cells express MHC II much less than the normal cells, particularly in humans, which causes SEC2 to lack the ability to recognize the tumor targets. Therefore, it is important to develop an appropriate method to allow SEs to specifically as well as efficiently recognize tumor cells for therapeutic purposes.

Immunotoxins consisting of a targeting molecule linked to a cytotoxic agent have been widely used as therapeutic agents for cancers (Kreitman 2003). The variable domains of antibodies or genetically engineered antibodies are usually used for targeting purposes. Recently, scFvs have drawn great attention for their tumor-targeting effect because of their small size suitable for drug development. Like many other tumor-associated antigens, Her2/neu, also known as c-erbB-2, is an oncogene product localized on tumor cell surface. It has been documented that this oncogene product is overexpressed in human breast and ovarian cancers by 25–30% (Slamon et al. 1989; Berchuck et al.1990). In the last decade, anti-Her-2 scFvs have been used widely as an effective vehicle for targeting treatment to HER-2 overexpressing tumor (Marshall and Marks 2001; Schmidt et al. 2001; Lisan et al. 1999; Altenschmidt et al. 1997).

In this publication, we report our recent study on the construction and characterization of a new immunotoxin, in which an HER-2-specific scFv fragment was fused with SEC2. This fusion immunotoxin has been successfully expressed and characterized. The experimental results showed that the constructed fusion immunotoxin retains anti-HER-2 scFv specificity as well as exhibit SEC2 bioactivity. In comparison with the native SEC2, the constructed fusion immunotoxin demonstrates enhanced antitumor activity against HER-2 overexpressing tumor in vitro.

X. Ming-Kai · Z. Cheng-Gang
Institute of Applied Ecology,
Chinese Academy of Science,
Shanyang, 110016, China

X. Ming-Kai
Graduate School of The Chinese Academy of Science,
Beijing, 100039, China

X. Ming-Kai (✉)
72 Wenhua Road,
P.O. Box 417 Shenyang,
110016, People's Republic of China
e-mail: mingkaixu@yahoo.com.cn
Tel.: +86-24-83970378
Fax: +86-24-83970300

Materials and methods

Cell lines and bacteria strain

Human breast cancer cells SK-Br-3 and MCF-7 provided by Wuhan Tongji Medical University were cultured in RPMI 1640 supplement with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin. *Escherichia coli* DH5α strain in Luria–Bertani (LB) medium and *E. coli* AD494(DE3) in LB medium with 15 μg/ml kanamycin were purchased from Novagen Company (USA). SEC2-producing *Staphylococcus aureus* (ol65Z-1) strain in LB medium was provided by Shenyang Xiehe Pharmaceutical Group Company (China).

Vectors

Cloning vector pGEM-T was purchased from Promega (USA). Expression vector pET-32a(+) was purchased from Novagen. Anti-HER-2 scFv plasmid pCynML3.9 was kindly provided by Dr. James Marks of the University of California-San Francisco.

Gene clone of SEC2 by PCR

Total DNA was prepared from *S. aureus* cultured overnight in LB medium(Ausubel et al. 1995). Based on the protein sequences of SECs, as well as the DNA sequences of SEC1 and SEC3, two oligonucleotide primers were designed. These were primer P1 (sense primer): 5′-GAA TTC GAG AGT CAA CCA GAC CCT A-3′ and primer P2 (antisense primer): 5′-CTC GAG TTA TCC ATT CTT TGT TGT A-3′. Both oligonucleotides were extended at their 5′ ends containing recognition sequences for endonucleases *Eco*RI and *Xho*I (underlined above). The total DNA was used as a template for polymerase chain reaction (PCR). PCR amplification was performed in the following procedure: 7 min at 94°C; followed by 30 cycles of 1 min at 94°C, 45 s at 55°C, and 1.5 min at 72°C; and then 10 min at 72°C. The PCR amplification products were purified by electrophoresis in a 1% Tris-acetate-ethylenediaminetetraacetic acid (TAE)–agarose gel and ligated into cloning vector pGEM-T by T4 DNA ligase to generate pGEM-T-sec2. The ligated products were transformed into *E. coli* DH5α (Sambrook et al. 2001) and sequenced from both ends by Sanger DNA sequencing.

Production of scFv DNA by PCR

Based on the DNA sequence of scFv-ml (pCynML3.9), two oligonucleotide primers were designed. These were primer F, containing an *Nco*I restriction site (sense primer): 5′-TTA TCC ATG GCC CAG GTG CAG CTG GTG CAG TCT-3′, and primer R, containing a *Not*I restriction site (antisense primer): 5′-TTC TGC GGC CGC ACC TAG GAC GGT GAC CTT GGT C A-3′. The plasmid DNA of pCynML3.9 was used as a template for PCR. PCR amplification was performed in the following procedure: 5 min at 94°C; followed by 30 cycles of 45 s at 94°C, 45 s at 55°C, and 2 min at 72°C; and then 10 min at 72°C. The PCR amplification products were purified by electrophoresis in a 1% TAE–agarose gel and digested by endonucleases *Nco*I and *Not*I, then gel-purified again.

Construction of the expression plasmid vector pET-32a-ml-l-sec2

Two oligonucleotide linkers were designed to ligate the gene of scFv and SEC2 to the fusion immunotoxin gene. These were linker L1, containing an *Not*I stick end (sense primer): 5′-G GCC GCA GGT GGA GGC GGT TCA GGC GGA GGT GGC TCT G-3′, and linker L2, containing a *Eco*RI stick end (antisense primer): 5′-AA TTC AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC TGC-3′. Annealing of these two oligonucleotide linkers was conducted for 10 min at 94°C, 10 min at 0°C, and 20 min at 72°C.

The expression vector plasmid pET-32a(+) DNA fragments digested by *Nco*I and *Xho*I, the scFv DNA fragments digested by *Nco*I and *Not*I, the *sec2* DNA fragments digested by *Eco*RI and *Xho*I, and the annealed linker were mixed with the proportion of 1:3:3:3 and were ligased overnight to construct fusion immunotoxin expression vector pET-32a-ml-l-sec2. The ligated products were transformed into *E. coli* AD494(DE3). The constructed fusion genes contain the fused thioredoxin (TRX) for high-level soluble expression and the His-tags for protein purification.

Expression and purification of fusion protein TRX-ML-L-SEC2

Single colony of AD494(DE3) with plasmid pET-32a-ml-l-sec2 was grown overnight at 37°C in LB medium with 100 μg/ml ampicillin and 15 μg/ml kanamycin. The cultures were diluted 50-fold in the same medium and grown at 37°C to an OD_{600} of 0.8–1.0. Expression of the fusion proteins was induced with 1.0 mM isopropylthiogalactoside (IPTG) for 5 h at 30°C. Cells were harvested by centrifugation for 10 min at 4°C and 4,000×*g* (r_{av} 8 cm), and the cell pellet was resuspended in ice-cold buffer A (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl). Cells were disrupted by sonication at 0°C and centrifuged for 30 min at 4°C and 10,000×*g* (r_{av} 8 cm). The supernatants were collected and loaded onto the Ni^{+}-saturated chelating sepharose column equilibrated with buffer A. After nonspecifically bound protein was washed off with buffer A containing 40 mM imidazole, the specifically bound protein TRX-ML-L-SEC2 was eluted with buffer A containing imidazole from 50 to 200 mM. The purity of the eluted protein was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining (Wang and Fan 2002).

Western blotting analysis of TRX-ML-L-SEC2

The purified proteins were analyzed with SDS-PAGE and then transferred into nitrocellulose membrane. The membrane was blocked with blocking solution [2% non-fat dried milk powder in phosphate-buffered saline (PBS) with 0.5% Tween 20] and then incubated with rabbit anti-SEC2 antibody diluted in blocking solution. Alkaline-phosphatase-labeled sheep antirabbit IgG was used as the secondary antibody, and specific binding was detected with nitro blue tetrazolium (NBT)/bromochloroindolyl phosphate (BCIP).

Enterokinase digestion of fusion protein

To remove the TRX and His-tag fused to the amino terminus, 1 mg purified fusion protein was digested with 20 U enterokinase by following the instruction of the Novagen recombinant enterokinase kits (Novagen). The mature fusion immunotoxin ML-L-SEC2 without His-tag was purified with Ni^{+}-saturated chelating sepharose column and buffer A containing 40 mM imidazole. The eluted protein was then desalted, concentrated, and determined by SDS-PAGE.

Binding specificity analysis of fusion immunotoxin with Western blotting

Harvested SK-Br-3 cells were lysed, and the lysate was analyzed with SDS-PAGE. After transfer, the nitrocellulose membrane was incubated subsequently with the purified fusion immunotoxin, rabbit anti-SEC2 antibody, and alkaline-phosphatase-labeled sheep antirabbit IgG. The binding specificity was then detected with NBT/BCIP.

Fusion immunotoxin stimulate PBMC proliferaton assay

Peripheral blood mononuclear cell (PBMC) from the blood of healthy donors was isolated by Ficoll density gradient centrifugation and aliquoted to 2×10^5 cells/well in 96-well plate in RPMI 1640 supplement with 10% FBS. A series of concentration of purified fusion protein TRX-ML-L-SEC2, fusion immunotoxin ML-L-SEC2, and standard SEC2 were added to triplicate wells. Bovine serum albumin (BSA) and phytohemagglutinin-P (PHA-P) were used as negative and positive controls, respectively. After 96 h of incubation at 37°C in 5% CO_2, 50 μl 5 mg/ml methyl thiazol tetrazolium (MTT) in PBS was added into each well, and the incubation was continued for another 4 h. The cells were collected by centrifugation for 10 min at room temperature and 1,000 rpm (r_{av} 8 cm). The pellet was redissolved in 120 μl DMSO for 20 min, and the absorbance was measured at a wavelength of 570 nm in a microplate reader.

In vitro antitumor activity of fusion immunotoxin

Tumor cells SK-Br-3 or MCF-7 were seeded in 96-well plate at a density of 1×10^4 cells/well in RPMI 1640 supplement with 10% FBS. Purified 100 ng/ml TRX-ML-L-SEC2, 100 ng/ml ML-L-SEC2, and 50 ng/ml standard SEC2 were added separately to triplicate wells, and the cells were incubated for 4 h before 2×10^5 PBMC cells were added. The blank wells (RPMI 1640 only), unsettled tumor cells control wells (tumor cells only), and PBMC releasing wells (PBMC and toxin protein) were used as control. The negative control was BSA.

The medium was removed from each well after incubation at 37°C for 72 h, and 150 μl fresh medium and 50 μl 5 mg/ml MTT in PBS were added into each well. Incubation was continued for another 4 h. Cells were then collected by centrifugation for 10 min at room temperature and 1,000 rpm (r_{av} 8 cm). The pellet was redissolved in 120 μl DMSO for 20 min, and the absorbance was measured at a wavelength of 570 nm in a microplate reader. The antitumor activity was calculated using the following formulation:

Tumor growth inhibition (%)=100-[(the OD_{570} of protein-treated cells well - the OD_{570} of PBMC-releasing wells)/(the OD_{570} of unsettled tumor cells control wells - the OD_{570} of blank control wells)]×100.

Results

Cloning and sequencing of SEC2

The *sec2* PCR amplificants were analyzed with electrophoresis in a 1% TAE–agarose gel (Fig. 1a). About 720-bp product was inserted into pGEM-T vector. A colony, designated pGEM-T-sec2, was verified by double digestion of *Eco*RI and *Xho*I (Fig. 1b). The verified colony was sequenced, and the result was translated to a protein sequence which is in accordance with the data of P34071 in GenBank. The DNA sequence was submitted to GenBank and the Accession number AY450554 has been assigned.

Construction of the expression vector plasmid pET-32a-ml-l-sec2

Anti-HER-2 scFv gene of about 770 bp has been amplified by PCR and analyzed with electrophoresis in a 1% TAE–agarose gel (Fig. 2a). The gene was linked with DNA linker, SEC2 gene, and pET-32a(+) plasmid to construct expression vectors pET-32a-ml-l-sec2 for protein TRX-ML-L-SEC2 (Fig. 2c). The coexpressed TRX at the N terminus of the expressed fusion protein is designed to enhance the solubility of the expressed fusion protein in cytoplasm. The constructed expression vector was verified by double digestion (Fig. 2b) and DNA sequencing.

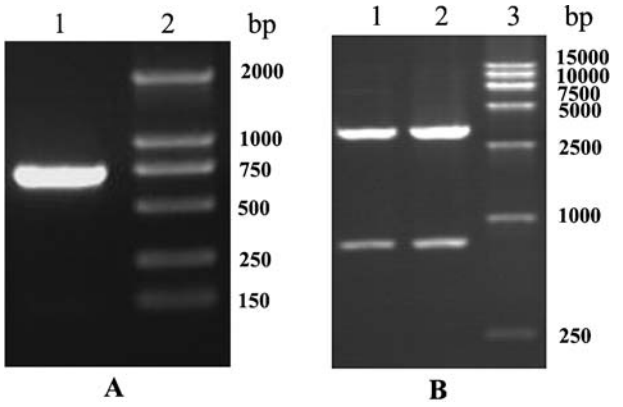

Fig. 1 The cloning of *sec2* gene by PCR from *S. aureus* genomic DNA. **a** PCR of *sec2* gene was performed, and the amplification products were analyzed with electrophoresis in a 1% TAE–agarose gel. *1* PCR products of *sec2*, *2* DL 2,000 DNA marker. **b** Verification of positive clone with pGEM-T-sec2 by double endonuclease digestion. *1* and *2* Result of double RE digestion of positive clone with pGEM-T-sec2 with *Eco*RI and *Xho*I, *3* DL 15,000 DNA marker

Expression and purification of fusion protein TRX-ML-L-SEC2

Expression of TRX-ML-L-SEC2 was induced with 1 mM isopropyl-β-D-galactopyranoside (IPTG) for 5 h at 30°C. After sonication and centrifugation, the bacterial supernatants were collected, and the pellets were resuspended in buffer A with one tenths of the supernatant volume. SDS-PAGE data showed the expected proteins of about 72 kDa in the lysates of the bacteria (Fig. 3a). After purification with Ni^{+}-saturated chelating sepharose, the fusion protein shown as a single band on SDS-PAGE was of purity of more than 95% (Fig. 3b). The yield of purified fusion protein was about 15 mg/l of original bacterial culture. Western blotting data showed that the antigenic activity of the purified fusion proteins could be recognized by rabbit anti-SEC2 antibody (Fig. 3c).

Enterokinase digestion of fusion protein and special binding analysis of fusion immunotoxin

The TRX-His tag fused to the amino terminus of the purified fusion proteins was removed by complete enterokinase digestion (Fig. 2c). After purification, the matured fusion immunotoxins ML-L-SEC2 of 60 kDa was shown as a single band on SDS-PAGE (Fig. 4a).

The HER-2 receptor of 185 kDa on HER-2 overexpression cells such as SK-Br-3 cell membrane was referred to as p185 protein. Western blotting showed that purified fusion immunotoxin could bind specifically to p185 protein (Fig. 4b).

Fusion immunotoxin stimulate PBMC proliferaton

The purified fusion protein and fusion immunotoxin were used to stimulate PBMC proliferation. BSA and PHA-P were used as negative and positive controls, respectively. The experimental data showed that the stimulation of PBMC proliferation by fusion protein and fusion immunotoxin is very efficient even at the concentration of 50 ng/ml, which is equivalent to that of the standard SEC2 (P>0.05) (Fig. 5). This indicated that the fused scFv frag-

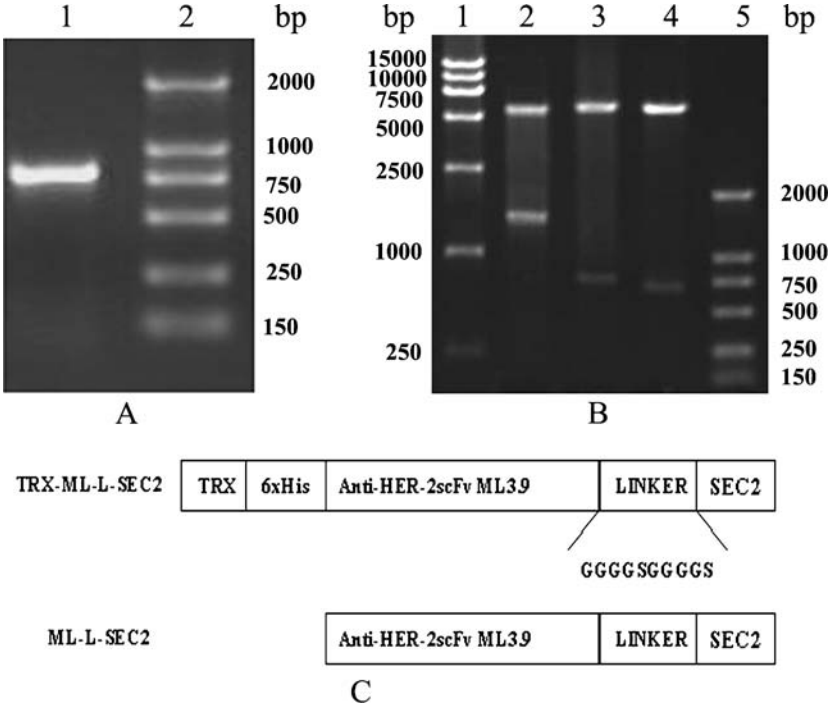

Fig. 2 Construction of the expression vector plasmid pET-32a-ml-l-sec2. **a** PCR of scFv-ml gene was performed, and the amplification products were analyzed with electrophoresis in a 1% TAE–agarose gel. *1* PCR products of scFv-ml, *2* DL 2000 DNA marker. **b** Verification of positive clone with pET-32a-ml-l-sec2 by double endonuclease digestion. *1* DL 15,000 DNA marker, *2* digestion of pET-32a-ml-l-sec2 by *Nco*I and *Xho*I, *3* digestion of pET-32a-ml-l-sec2 by *Nco*I and *Not*I, *4* digestion of pET-32a-ml-l-sec2 by *Eco*RI and *Xho*I, *5* DL 2,000 DNA marker. **c** The structures of fusion protein TRX-ML-LSEC2 and the mature fusion immunotoxin ML-L-SEC2 in which the fused TRX and His-tag were removed

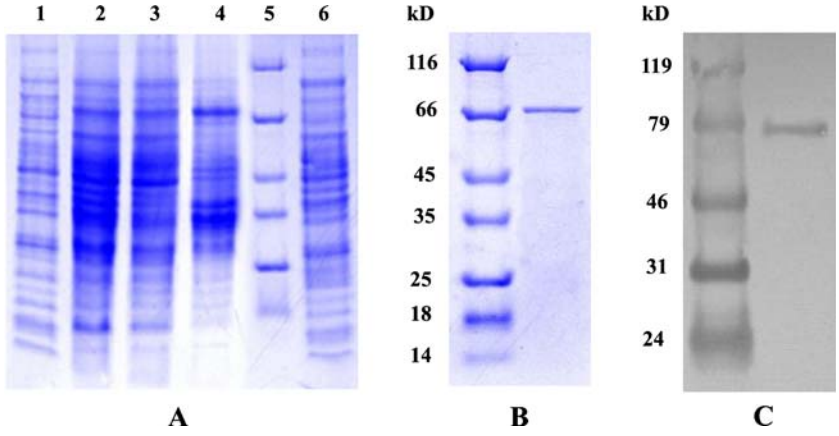

Fig. 3 The expression, purification, and Western blotting of fusion protein TRX-ML-L-SEC2. **a** The expression of TRX-ML-L-SEC2 induced with 1 mM IPTG at 30°C for 5 h. SDS-PAGE showed that the target protein with about 72 kDa could be detected in the lysate of *E. coli* AD494(DE3) transformed with pET-32a-ml-l-sec2 using Coomassie blue staining. *1* Uninduced AD494/pET-32-ml-l-sec2, *2* total protein expression of AD494/pET-32-ml-lsec2, *3* soluble expression of AD494/pET-32-ml-l-sec2, *4* inclusion expression of D494/pET-32-ml-l-sec2, *5* protein molecular weight marker (116, 66, 45, 35, 25, 18.4, and 14.4 kDa from the top band), *6* induced AD494/pET-32a(+). **b** About 1 mg of Ni-NTA purified TRX-ML-L-SEC2 was subjected to 12% SDS-PAGE and detected with Coomassie blue straining. **c** Western blotting of TRX-ML-L-SEC2. The transferred nitrocellulose membrane was incubated with rabbit anti-SEC2 antibody and alkaline-phosphatase-labeled sheep antirabbit IgG and detected with NBT/BCIP

ment did not inhibit the PBMC-proliferation stimulation activity of SEC2 in the constructed fusion immunotoxin.

In vitro antitumor activity of fusion immunotoxin

In comparison with standard SEC2, the matured fusion immunotoxin, ML-L-SEC2, demonstrated an enhanced growth inhibition activity against HER-2 overexpression tumor cells SK-Br-3. However, ML-L-SEC2 did not greatly inhibit the growth of MCF-7 cells (Fig. 6). These results indicate that the constructed immunotoxin has an antitumor activity specially targeted against HER-2 overexpression tumor cells. When compared to the undigested fusion protein, TRX-ML-L-SEC2, the matured immunotoxin was more effective in inhibiting the tumor cells SK-Br-3. This indicated that the fused TRX at the N terminus of scFv fragments retards the antibody activity of fusion immunotoxin.

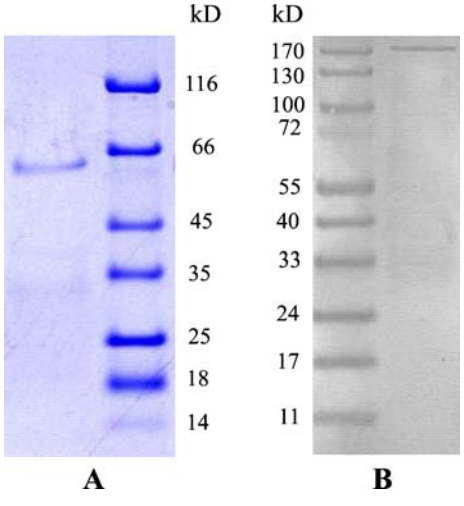

Fig. 4 Purification and special binding analysis of fusion immunotoxin B-L-SEC2. **a** After digestion by enterokinase, the matured fusion immunotoxin B-L-SEC2 was purified and analyzed by 12% SDS-PAGE and detected with Coomassie blue straining. **b** Western blotting showed the purified fusion immunotoxin ML-L-SEC2 specifically binds to HER-2

Discussion

As a superantigen, SEC2 can induce MHC II-independent immunostimulation, which is therapeutically relevant. In general, superantigens will direct T-cell cytotoxicity against MHC II-positive cells on which they are bound (Kalland et al. 1991). The feasible clinical application of superantigen-based antitumor strategies will require compromising MHC II binding capability. However, for humans, the expressed MHC II is localized on the surface of the normal cells and not tumor cells, which causes the lack of tumor targeting in tumor therapy. The ability of high doses of superantigen to cause T-cell anergy or deletion in vivo has also been documented (Wahl et al. 1993; MacDonald et al. 1991). Therefore, designing appropriate tumor-targeting vehicles to deliver SEs specifically to tumor cells could improve tumor therapy efficiency.

HER-2 receptor has been shown to play an important role in tumor development and progression. In particular, overexpression of HER-2 receptor has been correlated with poor clinical outcome in subsets of human malignancies (Hynes and Stern 1994). Due to the differential expression feature of such receptors, i.e., higher on the tumor cell surface and lower on normal tissues, therapeutic approaches specifically targeting HER-2 are expected to improve the clinical situation. Antibodies specifically against the extracellular domains of such receptors have

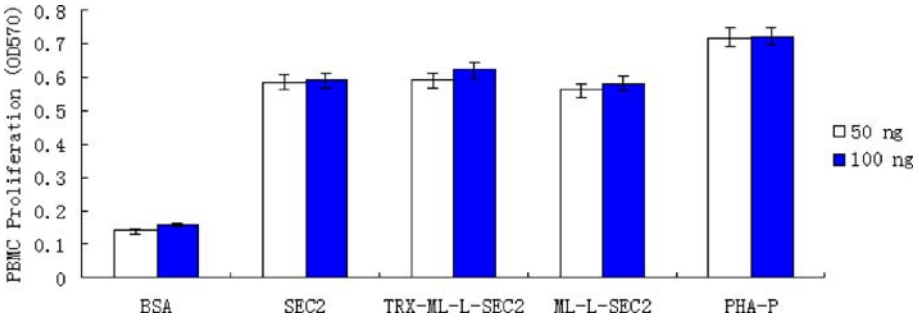

Fig. 5 Proliferation of PBMC by fusion proteins and fusion immunotoxins. PBMC was aliquoted to 2×10^5 cells/well in a 96-well plate, and different concentrations of purified TRX-ML-L-SEC2, ML-L-SEC2, and standard SEC2 were added to triplicate wells. BSA and PHA-P were used as negative and positive controls, respectively. After 96 h of incubation, 50 ml MTT in PBS was added into each well and incubated for another 4 h. The culture medium was removed, and 120 ml DMSO was added to each well. The OD570 was measured in a microplate reader

shown to interfere with signal transduction and are under therapeutical evaluation (Baselga and Mendelsohn 1994). For example, anti-HER-2 antibodies can be used to deliver highly cytotoxic moieties such as radionuclides, toxins, or chemotherapeutic agents to HER-2 overexpression tumor. In such circumstances, it is desirable to efficiently minimize the non-targeted toxicity to normal organs lacking HER-2 during the tumor-targeting process. scFvs hold the priority for such applications because of their small size. Therefore, linking anti-HER-2 scFvs to cytotoxic effector has a great potential for growth-inhibition of overexpressed tumor cells.

In this study, we describe the construction and expression of a novel fusion immunotoxin consisting of SEC2 and an anti-HER-2 scFv pCynML3.9. In designing the fusion immunotoxin, a number of considerations were proposed. As our long-term goal is the study of fusion immunotoxin for therapeutic use in humans, scFv pCynML3.9 was used because it is a humanized scFv, which would diminish human antimurine antibody (HAMA).

Previous studies suggested that the C terminus of SEC2 is important for its superantigen activity, whereas N terminus is important for its serological determinants (Hovde et al. 1990), and that the majority of recognition and binding functions of scFv is on its heavy chain (N terminus). Therefore, we decided to fuse the C terminus of scFv fragment to the N terminus of SEC2 to construct our fusion immunotoxin. A linker of ten amino acids consisting of glycin and serine was designed to link scFv and SEC2, which diminished the spatial interruption and maintained the native conformations of these two parts.

The results of this study proved the validity of our design. The presence of SEC2 at the carboxylic terminus of the scFv fragment does not hinder the ability of the combining site of the scFv to interact with Ag on the cell surface and does not diminish its binding ability. Similarly, the superantigen ability of the SEC2 in the fusion immunotoxin was not affected. The purified fusion immunotoxin showed a significantly enhanced growth-inhibitory effect on HER-2 overexpression tumor cells compared with standard SEC2.

As a special member of the SE family, SEC is a group of highly conserved proteins, including SEC1, SEC2, and SEC3. Protein sequences for the matured SEC1, SEC2, and SEC3 show the slight diversity on their amino terminus and the complete homology on their carboxyl terminus (Hovde et al. 1990; Bohach and Schlievert 1987; Passalacqua et al. 1993). Unfortunately, at this point, only protein sequences but no DNA sequence of SEC2 are available in GenBank, causing a difficulty in PCR primer design for the gene clone of SEC2. Based on the high conservation of DNA sequences between SEC1 and SEC3, as well as the protein sequences of SECs, we designed the PCR primers of SEC2. The result showed that the PCR product was indeed the gene of SEC2, and its sequence was submitted to GenBank with the Accession number AY450554.

In this study, expression vector pET32-a(+) was used for more fusion protein TRX-ML-L-SEC2 soluble expression

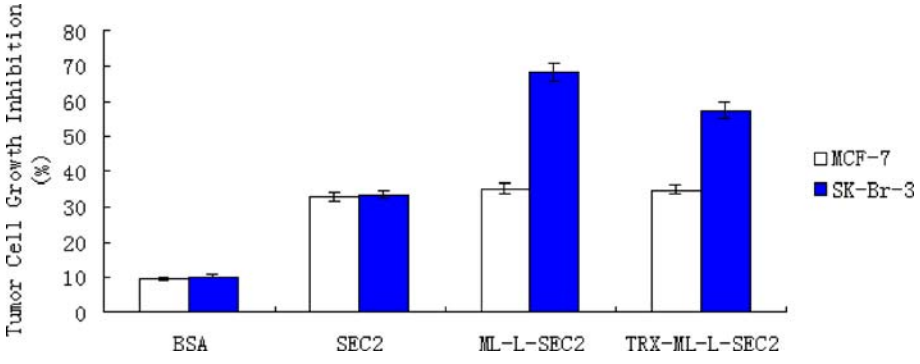

Fig. 6 Antitumor activity of fusion proteins and fusion immunotoxins by activating PBMC in vitro. Tumor cells were seeded in a 96-well plate at a density of 1×10^4 cells/well, and 100 ng/ml purified TRX-ML-L-SEC2 and ML-L-SEC2 and 50 ng/ml standard SEC2 (to get the same mole concentration) were added separately to triplicate wells to stimulate 2×10^5 PBMC cells to inhibit the growth of tumor cells

by fusing coexpressed TRX at the N terminus of expressed fusion protein. The result suggested that fused TRX hindered the antigen-recognizing and -binding function of scFv fragment in expressed fusion protein. We cleaved the fused TRX and His-tag fragment from the fusion protein by enterokinase digestion to obtain matured fusion immunotoxin ML-L-SEC2, which showed more effective growth-inhibition on HER-2 overexpression tumor cells in vitro.

In conclusion, a novel fusion immunotoxin ML-L-SEC2 was constructed and characterized. The mature fusion immunotoxin ML-L-SEC showed its antigen-binding specificity and antitumor targeting activity in vitro. These results in the current study are valuable for the further investigations of in vivo testing.

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