Cloning, purification and bioactivity assay of human CD28 single-chain antibody in *Escherichia coli*

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Abstract Engineered single chain antibodies have become a powerful source of immunotherapy against a wide range of diseases. Here, we present the generation of human CD28 single-chain antibody gene (CD28-ScFv), which contained variable fragments of heavy chain and light chain (VH and VL) of the anti-CD28 antibody, and a linking peptide (Gly4Ser)₃ inserted in the middle of VH and VL. The fused gene CD28-ScFv was successfully expressed in BL21 (DE3) cells and confirmed by western blotting assay. The molecular weight of CD28-ScFv was 43 kDa and the major fraction was expressed as an insoluble body. By dissolving the insoluble bodies, renaturing in vitro and purifying with a Ni-NTA affinity column, highly purified expression products of CD28-ScFv were obtained. This product could recognize and bind to CD28+ positive T cells. The proliferation capacity of peripheral blood T cells was increased by purified CD28-ScFv. In this study, we improved orthodox renaturing techniques by combining the dilution renaturation with phase gradient dialysis. With this

new method, highly purified CD28-ScFv products were developed and biological activity of the products was similar to that of the mouse monoclonal anti-human CD28 antibody.

Keywords CD28 · ScFv · Prokaryotic expression

Introduction

The agonist CD28 antibody has effects on T cells that are similar to those caused by natural ligand B7 when cross-linked with CD28, resulting in immunological effects, such as enhancing activation, proliferation and differentiation (Qiu et al. 2003). In recent years, more evidences indicate that tumor specific cytotoxic T lymphocytes can be amplified by a CD28 agonist monoclonal antibody or by incorporating a CD3 antibody and IL-2 cell factors (Michael et al. 1997; Chang et al. 1997). However, the mouse monoclonal antibody has some limitations in immunotherapy in the human body because of its immunogenic reaction even if the antibody from mouse is easily available. This research is warranted to further study since it is hard to deliver this kind of antibody to the target site because of its large molecular size and low penetration. To solve these problems, we reconstructed the mouse anti-human CD28 monoclonal antibody, which could specifically recognize CD28 molecule and initiate the costimulatory signal, resulting in

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activation and proliferation of T cells (Ju et al. 2003). In this study, we generated a single-chain anti-human CD28 antibody from the variable region of H-chain and L-chain of an anti-human CD28 antibody, with a small link peptide.

Also, single-chain antibodies are small molecules with a strong ability to penetrate tissue and a short half-life in blood plasma and have played an important role in the diagnosis and therapy of clinical disease (Winter and Milstein 1991; Grosse et al. 2003; Nieroda et al. 1995). In order to produce a large number of single-chain anti-human CD28 antibodies (CD28-ScFv) for research pertaining to immunology, biophysics, biochemistry and molecular biology, we developed genetic engineered CD28-ScFv in BL21 (DE3) cells. CD28-ScFv was highly purified by a modified degeneration, renaturation technology and affinity chromatograph protocol, producing a native protein with biological activity.

Materials and methods

Plasmids and strains

Plasmid pMD18-T/CD28VH and pMD18-T/CD28VL containing the H-chain and L-chain of mouse anti-human CD28 monoclonal antibody (clone 8G8A) respectively were from Medical Biotechnology Institute of Soochow University. The pET32a (+) vector and the bacterium BL21 (DE3) were purchased from Novagen (San Diego, CA) and Top10 cells were obtained from the Biochemistry & Molecular Biology Laboratory of Soochow University.

Enzymes, reagents and cell lines

Pyrobest DNA polymerase, LA Taq DNA polymerase, restriction enzymes *EcoRI*, *BamHI*, *HindIII*, *PstI*, T4 DNA ligase and other molecular biology reagents were purchased from Takara Bioengineering Company (Shiga, Japan). Plasmid mini-prep kits and gel extraction kits were from Invitrogen (Carlsbad, CA). Ni-NTA agarose was purchased from Qiagen (Valencia, CA) and FITC-labeled sheep anti-His IgG was obtained from Novagen. Mouse anti-human CD28 monoclonal antibody was provided by the Medical Biotechnology Institution of Soochow University (Qiu et al. 2001; Sun et al. 2005), and human CD28 gene transfected cells (CD28-T) were

obtained from Montpellier Gene Institute in France (Qiu et al. 2005). Human anticoagulated peripheral fresh blood was supplied by Suzhou Blood Center (Suzhou City, China).

Primers of FvH and FvL

All primer pairs were synthesized by Shenggong Bioengineering Technology Limited (Shanghai, China). FvH-A: 5'-GTC CTG CAG ATG GTG CAG CTT CAG CAG TCA GGA CCT GAG-3'; FvH-B: 5'-TCC GCC TCC GCT ACC TCC GCC GCC GGC TGA GGA GAC TGT GAG AGT GGT-3'; FvL-A1: 5'-GGA GGC TCT GGC GGA GGC GGT AGC GAC ATT GAG ATG ACC CAG TCT CCA-3'; FvL-B1: 5'-**ATG GTG GTG ATG GTG GTG** CCG TTT GGT TTC CAG CTT GGT GCC-3' (dashed area contains 6× His-tag sequence); FvL-A2: 5'-AGC TTA GGA GGT AGC GGA GGC GGA GGC TCT GGC GGA GGC GGT AGC-3'; FvL-B2: 5'-CTG GAA TTC TTA ATG GTG GTG ATG GTG GTG CCG TTT-3' (underlined sequence is *EcoRI* site and dashed area contains 6× His-tag sequence).

Amplification of FvH and FvL by polymerase chain reaction (PCR)

Plasmids pMD18-T/CD28VH and pMD18-T/CD28VL, with the variable region gene of H-chain and L-chain of mouse anti-human CD28 monoclonal antibody, were used as the template. We used Pyrobest enzyme and amplified the variable region gene, FvH and FvL1, of H-chain and L-chain of mouse anti-human CD28 monoclonal antibody using primers FvH-A and FvH-B, FvL-A1 and FvL-B1. The amplifying conditions were 94 °C denaturation for 1 min, 94 °C denaturation for 55 s, 58 °C annealing for 50 s, 72 °C extension for 55 s with 35 amplifying cycles, and a final 72 °C extension for 10 min. Then, the PCR products were excised by DNA Gel Extraction Kit after 1% agarose electrophoresis.

Using gel extracted product FvL1 as a template, FvL2 was amplified by LA Taq DNA polymerase with primers FvL-A2 and FvL-B2, with the following conditions: 94 °C denaturation for 1 min, 94 °C denaturation for 55 s, 58 °C annealing for 50 s, 72 °C extension for 55 s, repeated for 35 cycles, and a final 72 °C extension for 10 min. The PCR products

were extracted by gel extraction kit after 1% agarose electrophoresis.

Formation of CD28-ScFv gene and construction of the CD28-ScFv expression vector

Using FvH and FvL2 as templates, we linked the H-chain and L-chain of CD28 by a flexible peptide with the method of tri-primer (TP)-PCR. Then, FvH and FvL2 genes were assembled with LA Taq DNA polymerase ligated at the synthesized *Eco*RI and *Eco*RV double restriction sites. The reaction was divided into two steps. First, we added primers FvH-A and FvL-B2 after 94 °C pre-denature for 1 min, and 10 cycles of 94 °C denaturation for 45 s, 50 °C annealing for 45 s and 72 °C extension for 1 min. Then, after 35 cycles of: denaturation at 94 °C for 1 min, 60 °C annealing for 1 min, 72 °C extension for 2 min and final extension at 72 °C for 10 min, the PCR product was assembled into the CD28-ScFv gene successfully. The segment was extracted by Gel Extraction Kit. Construction strategy is shown in Fig. 1.

The CD28-ScFv gene was inserted into the pET32a expression vector through the double restriction sites. The ligation product was transformed into competent Top10 cells with CaCl_2 (0.01 M) at 42 °C for 1.5 min. Then the cells were cultured on the solid LB medium plate at 37 °C overnight. The positive

clone was identified by double digestion with *Eco*RI and *Eco*RV, and confirmed by PCR method and DNA sequencing (ABI3730, Invitrogen, Shanghai).

Expression of CD28-ScFv protein

BL21 cells transformed with recombinant expression vectors were cultured at 37 °C, 250 rpm overnight. On the next day, the culture was seeded into fresh Luria–Bertani (LB) medium at 1:100 until OD_{600} reached 0.5, and then protein expression was induced with 0.1 mmol/L isopropyl-beta-D-thiogalactopyranoside (IPTG) for 4 h at 30 °C, 250 rpm. The bacteria bodies were harvested and were ultrasonicated and disrupted with a liquid nitrogen freeze/thaw cycle. The cell lysate was incubated in an ultrasonic ice bath (150 W $\times$ 100 times $\times$ 2 s) and then further disrupted by three freeze/thaw cycles in liquid nitrogen. After that, the lysate was centrifuged at 12,000 rpm for 20 min at 4 °C, and the pellet was washed twice with cytorrhcytes washing buffer to obtain pure cytorrhcytes proteins.

Degeneration and refolding of CD28-ScFv and purification by Ni-NTA affinity column

The pure cytorrhcytes were resuspended in denaturing buffer and incubated overnight at 4 °C, and dithiothreitol (DTT) was added to a final concentration of 10 mmol/L. Then the sample was divided into 12 groups, according to the different degeneration and desalination conditions (Table 1). After that, the sample was dialyzed in slow dialysis solution to refold the correct three-dimensional structure of CD28-ScFv, followed by three times in dialysis buffer without urea, 45 min for each dialysis to regenerate the native ScFv. Finally, the protein was purified by Ni-NTA affinity column according to the instruction (Novagen, Darmstadt, Germany).

Western blotting assay for CD28-ScFv

The protein samples were loaded on to a 12% SDS-PAGE gel (80 V for 5% gel and 150 V for 12% gel) and transferred onto a nitrocellulose membrane (100 V, 2 h at 4 °C). The membrane was blocked overnight in 5% skim milk in phosphate buffered saline/Tween-20 (PBS-T) buffer at 4 °C, and then was incubated with mouse anti-His-tag antibody (1:1,000) at room

Fig. 1 Construction strategy of recombinant expression vector pET32a CD28-ScFv

Table 1 Groups of CD28-ScFv in different degeneration conditions

Groups	Degeneration	Slow dialysis	Quick dialysis	Desalination (yes or no)
1	Urea 3 mol/L; Tris-HCl 25 mmol/L pH 8.7; glutathione (reduced) 0.1 mol/L; glutathione (oxidized) 0.1 mmol/L; L-Arg 0.4 mol/L; 48 h	Urea 0.25 mol/L; Tris-HCl 25 mmol/L pH 8.0; NaCl 0.15 mol/L; 24 h	Tris-HCl 25 mmol/L pH 8.0; NaCl 0.15 mol/L; 3 times, 45 min per time	Yes No
2	Same as above	Urea 2 mol/L; Tris-HCl 25 mmol/L pH 8.0; NaCl 0.15 mol/L; 24 h	Same as above	Yes No
3	Same as above	Urea 3 mol/L; Tris-HCl 25 mmol/L pH 8.0; NaCl 0.15 mol/L 5 h; Change dialysis density of Urea. Do dialysis at 2.5, 2, 1.5 mol/L for 5 h, respectively	Same as above	Yes No
4	Urea 3 mol/L; Tris-HCl 25 mmol/L pH 8.7; glutathione (reduced) 0.1 mol/L; glutathione (oxidized) 0.1 mol/L; L-Arg 0.4 mol/L; 10% glycerol; 48 h	Urea 0.25 mol/L; Tris-HCl 25 mmol/L pH 8.0; NaCl 0.15 mol/L; 24 h	Same as above	Yes No
5	Same as above	Urea 2 mol/L; Tris-HCl 25 mmol/L pH 8.0; NaCl 0.15 mol/L; 24 h	Same as above	Yes No
6	Same as above	Urea 3 mol/L; Tris-HCl 25 mmol/L pH 8.0; NaCl 0.15 mol/L 5 h; change dialysis density of urea. Do dialysis at 2.5, 2, 1.5 mol/L for 5 h, respectively	Same as above	Yes No

temperature for 2 h. After being washed in PBS-T three times for 10 min each, sheep anti-mouse IgG labeled with alkali phosphatase was added and incubated for 1 h at room temperature. After that, the membrane was washed for 10 min three times with PBS, and the protein bands were detected with NBT/BCIP reagents according to the protocol (Roche, Indianapolis, IN).

CD28-ScFv binding to membrane-bound CD28

The cultured CD28-T cells were collected, washed with PBS and divided into flow cytometry analysis tubes at 5×10^5 cells per tube, then the purified CD28-ScFv was added at a concentration of 1 µg per tube and incubated for 30 min at 4 °C. After washing, goat anti-His antibody labeled with FITC was added and incubated for 30 min at 4 °C. Finally, the stained cells were analysed by flow cytometry (488 nm, Altra, Beckman Coulter, Fullerton, CA).

Proliferation effect of PBTC with CD28-ScFv and anti-human CD3 monoclonal antibody

Peripheral blood mononuclear cells (PBMC) were isolated from fresh anticoagulant blood by Ficoll lymphocyte separation solution. Peripheral blood T cells (PBTC) were obtained after E rosette test of sheep red blood cells (RBCs). We adjusted the PBTC concentration to 1×10^6 cells/mL and spread the cells into a 96-well plate coated with anti-human CD3 monoclonal antibody (MAb) (1 µg/mL) at 100 µL/well. After that, CD28-ScFv was added to final concentration of 5, 2.5 and 1 µg/mL, respectively. Next, we placed the 96-well plate into an incubator set at 37 °C, 5% CO₂, and incubated for 72 h; and in the final 6–8 h, MTT solution (5% w/v, 20 µL/well) was added. Then, proliferation activity was detected by automated immunoanalyzer at OD₅₇₀, and mouse anti-human CD28 MAb was served as a positive control.

Results

Generation of single-chain anti-human CD28 antibody gene (CD28-ScFv)

The VH and VL DNA fragments of anti-human CD28 antibody were successfully amplified from pMD18-T/CD28VH and pMD18-T/CD28VL with

primers FvH-A & FvH-B and FvL-A1 & FvL-B1, respectively. The size of amplified FvH DNA was 381 bp as expected, and the correct sequence was confirmed by sequencing. The size of FvL1 DNA was 345 bp as expected, without any mutations. Using FvL1 as a template and FvL-A2 & FvL-B2 as primers, the FvL2 fragment was successfully amplified and was consistent with the theoretical size.

Using FvH and FvL2 as templates, a 750 bp DNA fragment was obtained by splicing FvH and FvL2 with LA Taq DNA polymerase. The sequencing results showed that anti-human CD28-ScFv gene was free of mutations, as was the connecting peptide in middle of FvH and FvL and the His-tag on the 3' end (Fig. 2).

Construction of recombinant expression vector pET-32a-CD28-ScFv

The purified CD28-ScFv gene fragment was digested with *Eco*RI and *Eco*RV and cloned into the pET-32a (+) transfer vector, and the ligation product was transformed into competent Top10 cells. The positive clone was identified by enzymatic digestion and PCR amplification (Fig. 3).

The pET-32a-CD28-ScFv vector was sequenced in two directions around CD28-ScFv. The sequence of CD28-ScFv was correct, and measured 753 bp, including VH (345 bp), VL (320 bp), connecting peptide (45 bp) and the 6× His-tag sequence added on the 3' end of CD28-ScFv (Fig. 2).

Expression and purification of CD28-ScFv

Escherichia coli BL21 (DE3) cells, containing expression vector pET32a-CD28-ScFv, were induced with IPTG at 37 °C for 4 h, and a 43 kDa protein corresponding to the size of CD28-ScFv was highly expressed. The major fraction of CD28-ScFv existed as insoluble body (Fig. 4a). The insoluble body contained relatively pure CD28-ScFv, of more than 90% of total protein.

Finally, the CD28-ScFv insoluble body was denatured by guanidinium hydrochloride, renatured in dialysis buffer, and the regeneration protein was purified by Ni-NTA affinity column. The molecular weight of the target protein was about 31–43 kDa, consistent with the theoretical value. Furthermore, the nature of CD28-ScFv was confirmed by western blotting (Fig. 4b).

Fig. 2 Nucleotide sequence and its deduced amino acid sequence of the anti-CD28-ScFv. The *shadowed* part represents the Linker, the *underlined* part is the 6× His-tag

Denaturation, renaturation and purification of CD28-ScFv

The major fraction of CD28-ScFv existed as cytotocytes in BL21 cells. In order to raise the yield and activity of such purified products, several methods of purification of CD28-ScFv from cytotocytes were tested. It was clear that arginine increased the yield of purified CD28-ScFv. In contrast, renaturation without arginine yielded only 10% of protein; while adding arginine raised the yield to 21%. In all six different denaturation conditions, single-chain anti-

human CD28-ScFv was isolated with high purity, and there were no differences in the yield of soluble CD28-ScFv protein (Fig. 5). The six groups of CD28-ScFv from the Ni-NTA affinity column were desalted and concentrated by centrifugal ultrafiltration equipment.

Conjugation of CD28-ScFv and cellular membrane model CD28

The binding activity of CD28-ScFv to CD28-T cells was dependent on renaturation conditions, as

Fig. 3 Identification of recombinant expression vector pET32a with anti-CD28-ScFv. *M*: DNA marker; *1*: digested by *EcoRI* + *EcoRV*; *2*: identified by PCR

determined by FACS assay. The salinity had a direct impact on the specific binding of antigen to antibody. The positive binding rate of CD28-ScFv to CD28-T cells was higher (up to 69.6%) after desalination compared to non-desalination of CD28-ScFv (around 17.3%) (Fig. 6).

Proliferation effect of PBTC with CD28-ScFv and CD3 MAb

The first signal needed in T cell activation was provided by the CD3 MAb, and the second signal needed in T cell activation was provided by CD28-ScFv. Results of MTT assay showed that the proliferation effect of PBTCs was induced by 5 µg/mL CD28-ScFv corresponded to 41.8% of the mouse CD28 MAb with the same concentration ($1.02/1.95 \times 100\%$) (Fig. 7).

Fig. 4 Expression and purification of CD28-ScFv from bacteria. **a** Coomassie blue staining; **b** western blotting with anti-His6 antibody

Fig. 5 Purification of CD28-ScFv with different renaturing conditions. *M*: Protein standard molecular weight; *1* ~ *6* represent groups 1 ~ 6 of purified proteins as shown in Table 1 (All loaded proteins were with desalination)

Discussion

When many hybrid proteins are expressed, renatured proteins often react with other hybrid proteins, causing sediments that greatly affect the ability of the protein to renature. In this paper, the purity of cytotocytes reached above 90% with the ultrasonic treatment and liquid nitrogen freeze/thaw method, which also contributed to renaturation. Moreover, the cysteine residues in CD28-ScFv peptide chains could form disulfide bonds (S–S) in cytotocytes. In order to denature CD28-ScFv fully, we added DTT into the denaturing buffer (Baryshnikova et al. 2005). It is interesting that arginine can cause the protein yield increasing to 21%, compared to 10% in the preliminary experiment. Moreover, during the renaturation, proteins in solution can get together easily and form sediments. However, 0.4 mol/L arginine can weaken the reaction among proteins and hamper

Fig. 6 Analysis of conjugation of CD28-ScFv and CD28-T cells by FACS. **a** Negative control (FITC-labeled mouse IgG); **b** positive control (FITC-labeled mouse anti-human CD28); **c** renaturing 5th group of CD28-ScFv (not desalted) (first antibody: CD28-ScFv, second antibody: FITC-labeled sheep

anti-His IgG); **d** renaturing 5th group of CD28-ScFv (desalted) (antibodies are the same as in **c**); **e** renaturing 6th group of CD28-ScFv (not desalted) (antibodies are the same as in **c**); **f** renaturing 6th group of CD28-ScFv (desalted) (antibodies are the same as in **c**)

Fig. 7 Effects of CD28-ScFv on proliferation of PBTC. **A**: control; **B**: mouse anti-human CD28 MAb (5 µg/mL); **C**: CD28-ScFv (5 µg/mL); **D**: CD28-ScFv (2.5 µg/mL); **E**: CD28-ScFv (1.25 µg/mL)

the concentration of folding intermediate (Tsumoto et al. 2005; Baynes et al. 2005).

Glycerine is a typical kind of penetrant or osmolyte. It can help proteins fold to an active conformation, speed up their folding velocity, and stabilize proteins and raise protein yield (Dong et al. 2004). The presence of glycerine played an important role in CD28-ScFv activity. It is clear that activity of CD28-ScFv was higher in groups 4, 5 and 6 compared to that of groups 1, 2 and 3. This indicates that glycerine can definitely contribute to protein folding and stabilization. Furthermore, the activity of purified CD28-ScFv in group 6 was much higher than

that of groups 4 and 5, which were treated with the traditional purification methods.

In the 6th group, we made use of a recently developed commercial renaturing column. In recent years, many hydrophobic interaction chromatography (HIC) columns have been developed to prevent proteins from concentrating, but they have produced proteins with faulty folding. Sometimes, the protein yield was very high, but proteins with true biological functions were less abundant than those from the dilute reannealing method. Furthermore, research showed that the protein folding midpoint is the moment when a protein is in a dynamic pilot process comprised 50% folded and 50% unfolded molecules at a certain urea concentration (Dong et al. 2004). To get better renaturation, we need to let proteins go through this folding midpoint in a definitive way. During the experimental protocol, if we remove the protein denaturant too quickly and let proteins pass this folding midpoint quickly, the denatured proteins will not have enough time to fold in an active configuration, and many disulfide bonds will not match with each other. But if we gradually decrease the urea density in the reannealing liquid and allow enough time for reannealing at each grade of urea density, the proteins will recover and achieve their active conformation, approaching in vivo status. When expressing exogenous genes in *E. coli*, it is

essential to find optimal reannealing conditions and use proper reannealing methods for the purification of expressed proteins in cytocytes to obtain proteins with biological activity.

In recent years, research on single-chain antibody fragments (ScAb) has been very popular. It was reported that an anti-human ScAb with the correct folding style of apolipoprotein A-I (apo A-I) protein in plasma has a binding activity of antigenic specificity, which is 1/700 of its parent MAb (Cho et al. 2000). Choi and colleagues prepared and purified ScAb from anti-mycotoxin deoxy nivalenol (DON) proteins, the activity of which was 1/42 of its parent MAb (Choi et al. 2004). Recently, Yang et al. (2005) obtained ScAb of the anti-human HBsAg PreS1 segment with the correct folding conformation, and its activity was 1/60 of its parent MAb.

In this research, after obtaining CD28-ScFv at different reannealing conditions, we used immunofluorescence and flow cytometry to analyze the binding between CD28-ScFv and CD28 on the CD28-T surface. The results showed that the positive binding rate of CD28-ScFv to CD28-T was dependent on denaturation and renaturation conditions. The binding rate reached its peak at 69.6% in the experiment conditions of group 6. This indicated that glyoxalin in the protein eluant can impact the binding ability of CD28-ScFv and its antigen, but the mechanism of such binding is under investigation. On this basis, we used CD3 MAb to coat cell culture plates and analyzed the proliferation effect of PBTCs with CD28-ScFv. The results showed that the activation and proliferation of PBTC needed two signals, one of which was the first signal to bind CD3 molecules on the T cell with CD3 MAb and provide T cell activation; the other was the second signal which bound T cell CD28 with CD28-ScFv and provided T cell activation. In this research, we set up different experimental groups of CD28-ScFv with a final concentration of 5.0, 2.5 and 1.25 µg/mL, respectively. The flow cytometry results showed that the binding affinity of ScFv to CD28 antigen was lower than that of the parent antibody. The MTT results indicated that the proliferation effect of CD28-ScFv on PBTC is also weaker than that of mouse CD28 MAb at the same concentration.

In this study, we obtained CD28-ScFv with good biological activity for the first time by genetic engineering technology. It is promising that such a

proliferation effect of the antigen anti-PBTC will play a potential applied value in future tumor adoptive immunotherapy.

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