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A strategy for high-level expression of a single-chain variable fragment against TNFα by subcloning antibody variable regions from the phage display vector pCANTAB 5E into pBV220

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ABSTRACT

A phage display single-chain variable fragment (scFv) library against TNFα was constructed using a recombinant phage antibody system (RPAS). The cloned scFv gene was introduced into the phage display vector pCANTAB 5E and expressed in *Escherichia coli* (*E. coli*) with a yield of up to 0.15 mg/l of total protein. With the attempt to improve the expression level of TNF-scFv, a strategy was established for subcloning the scFv gene from pCANTAB 5E into the plasmid pBV220. Under the control of a highly efficient tandem P_RP_L promoter system, scFv production was increased to 30% of total protein as inclusion bodies. After extraction from the cell pellet by sonication, the inclusion bodies were solubilized and denatured in the presence of 8 M urea. Purification of denatured scFv was performed using nickel column chromatography followed by renaturation. The purity and activity of the refolded scFv were confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), Western blotting and by an enzyme-linked immunoabsorbent assay (ELISA). The results reveal that the overall yield of bioactive TNF-scFv from *E. coli* flask cultures was more than 45 mg/l culture medium and 15 mg/g wet weight cells. The renatured scFv exhibited binding activity similarly to soluble scFv. In conclusion we developed a method to over-express TNF-scFv, which have biological function after purification and renaturation.

Introduction

In recent years, genetic engineering techniques have been widely used to develop antibody-like molecules for therapeutic and diagnostic purposes. The expression of recombinant antibodies and their fragments has become an area of interest in biotechnology [1–3]. By using the phage display system, antibody fragments can be displayed on the surface of phage and fused to a minor coat protein III and specific antibodies can be obtained by several rounds of antigen selection [4]. There are many forms of antibody derivatives, such as Fab[1], $F(ab)_2$ and scFv with a linker peptide that connects V_H and V_L domains [5]. Many studies have concentrated on scFv due to its more rapid blood clearance and better tumor penetration in comparison with larger antibodies forms mentioned above. Therefore scFv represents potentially useful molecules for targeted delivery of drugs, toxins or radionuclides to tumors [6–8]. Consequently the potential uses of scFv in many areas of medicine drive

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[1] *Abbreviations used*: 4-CN, 4-Chloro-1-naphthol; BSA, bovine serum albumin; Fab, antigen-binding fragment; FBS, fetal bovine serum; HRP, horseradish peroxidase; HBsAg-scFv, anti-HBsAg scFv; IPTG, isopropyl-β-D-thio-galactopyranoside; mAb, monoclonal antibody; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; Ni–NTA, Ni^{2+}–nitrilotriacetic acid; pfu, plaque forming unit; PBS, Phosphate buffered saline; PMSF, phenylmethyl sulfonyl-fluoride; rhTNFα, recombinant human TNFα; rhTRAIL, recombinant human TNF-related apoptosis-inducing ligand; RPAS, recombinant Phage Antibody System; TMB, 3,3′,5,5′-tetramethylbenzidine; TNF-scFv, anti-TNFα scFv; VH, variable region of heavy chain; VL, variable region of light chain.

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the search for new alternative methods for large-scale production for both research and clinical purposes.

Recombinant Phage Antibody Systems (RPAS) are now commonly used for the display and production of scFv [9,10]. The phagemid vectors, such as pCANTAB 5E, have the advantage of efficient ligation–transformation to allow creation of libraries of larger size that are relatively easy to manipulation genetically to introduce special features that might be advantageous [11]. However, in spite of the wide use of RPAS, their use is hampered by the high cost of IPTG and lower yields of scFv. With regard to economic impact on the health care system, high-level expression systems for the production of scFv may be of crucial importance. One feasible method to overcome these shortcomings is combining the phage display vector pCANTAB 5E with a highly efficient expression system such as pBV220. This strategy makes good use of the selection of specific scFv by pCANTAB 5E and the over-expression of large amounts scFv by pBV220 vector [12]. A scFv against TNFα has been selected as a model system for this study. In current study we report successful construction of a phage display scFv library against TNFα using RPAS. Antigen specific scFv was selected by phage ELISA. The expression and purification of scFvs using two vectors, pCANTAB 5E and pBV220, are described. We demonstrated that the yield of scFv protein can be substantially increased using the pBV220 expression vector. This engineered scFv exhibits similar bioactivity to scFv expressed in its soluble functional form using pCANTAB 5E.

Materials and methods

Plasmids, strains, cells and reagents

Phagemid pCANTAB 5E was purchased from Amersham Biosciences (Germany). Plasmid pBV220 was constructed by Professor Zhang Zhi-qing. *Escherichia coli* host strain BL21 (DE3) was purchased from Novagen (Germany). *E. coli* TG1, HB2151 and M13 KO7 helper phage were purchased from Amersham Biosciences (Germany). The murine fibrosarcoma cell line, L929, was cultured in RPMI 1640 medium supplemented with heat-inactivated FBS 10% (v/v), penicillin (100 U/ml) and streptomycin (0.1 mg/ml) at 37 °C in a humidified incubator under a 5% CO_2 atmosphere. His-Tag monoclonal antibody was purchased from Novagen (Germany). HRP-conjugated mouse anti-M13 phage antibody and anti-E Tag antibody were from Amersham Biosciences (Germany). HRP-conjugated goat anti-mouse IgG was purchased from Promega (USA). Anti-TNFα mAb was purchased from PeproTech. Inc. (USA). HBsAg-scFv was constructed and stored in our laboratory. The Trizol reagent, restriction enzymes, T4 DNA ligase and Taq DNA polymerase were obtained from TaKaRa (Japan). Recombinant human (rh) TNFα, rhTRAIL was purchased from PeproTech. Inc. (USA). Complete and incomplete Freund's adjuvant, GSH, GSSG, 4-CN, TMB, PMSF and actinomycin D were from Sigma (USA). Centrifugal filter was purchased Millipore (USA). HiTrap anti-E Tag column was purchased from Amersham Biosciences (Germany). Ni–NTA agarose was purchased from Qiagen (Germany). Ninety six-well plates were obtained from Nunc (Denmark).

Mice immunization

Five BALB/c female mice (6–8 weeks, Academy of Military Medical Science; Beijing, China) were immunized intraperitoneally with 5 μg/injection of rhTNFα in 100 μl PBS mixed with 100 μl complete Freund's adjuvant. Immunizations were repeated at days 15 and 30 with incomplete adjuvant. On day 40 lymphocytes were harvested from spleen and used for total RNA preparation.

cDNA synthesis and phage display library construction

Total RNA was prepared from lymphocytes using the Trizol reagent and was reverse-transcribed into cDNA using random primers according to standard procedures. Primer sets (Amersham Biosciences, Germany) were used to PCR-amplify the variable heavy (V_H) and light (V_L) chain regions of the immunoglobulins expressed in the immunized mice. These cDNA fragments were combined with a $(Gly_4Ser)_3$ linker to generate scFv and inserted into phage-displayed vector pCANTAB 5E. The recombinant phagemids were used to transform competent *E. coli* TG1 cells to express phage-displayed scFv.

Selection of antigen specific scFv antibody

A phage-displayed scFv library was panned against immobilized rhTNFα, using 200 ng of protein for coating, 0.05% (v/v) Tween-20 in PBS (PBST) for washing, and 0.1 M HCl–glycine, pH 2.2, for elution. Individual clone randomly picked after the third panning were rescued on a small scale, and their specific binding to rhTNFα was determined by phage ELISA. Briefly, phage antibodies containing supernatants with 5% BSA were added into 96-well plates coated with 200 ng rhTNFα and incubated for 2 h at room temperature. The bound phages were detected by incubation with HRP-conjugated mouse anti-M13 antibodies for 1 h. The ELISA was developed for 10–20 min with a TMB-solution according to the supplier's instructions (Sigma). Wells directly coated with 10^9 M13 KO7 phages were used as positive control. Clones exhibited signals of 50% greater than of those of the positive controls were called strong binders. DNA samples of strong binders were selected and subjected to DNA sequencing analysis.

Expression and purification of pCANTAB 5E-scFv

To express TNF-scFv, phagemid pCANTAB 5E-scFv was transformed into the non-suppressor *E. coli* strain HB2151 with induction of 0.5 mM IPTG for 4 h in 2× YT medium. The cells were harvested by centrifugation at 4200*g* for 15 min and resuspended in 20 ml ice-cold buffer (0.5 M sucrose, 0.5 mM EDTA, 200 mM Tris–HCl, pH 8.0) [13]. Following 30 min of incubation on ice and centrifugation at 10,000*g* for 15 min, the supernatant was recovered as the periplasmic cell fraction and concentrated by ultrafiltration. Soluble antibodies were purified using an anti-E Tag column according to the supplier's protocol (Amersham Biosciences, Germany).

Construction of pBV220-scFv expression vector

For construction of pBV220-scFv, the forward primer 5′-AAGGATCCCATGCAGGTGAAACTG GTGGAGTCT-3′ and reverse primer 5′-GCGGATCCCTATTA gtggtggtggtggtggtg TCTTTTGA ATTC-3′ were used to amplify the pCANTAB 5 E-scFv to introduce *Bam*H I restriction sites (underlined) and six continuous histidines on the C-terminus of scFv (lowercase letters). The PCR conditions were as follows: 94 °C for 5 min, then 94 °C for 1 min, 55 °C for 1.5 min, 72 °C for 2 min for 35 cycles, then 72 °C for 10 min. The PCR product was digested with *Bam*H I and inserted into the bacterial expression vector pBV220 to give pBV220-scFv. The ligation products were transformed into *E. coli* BL21(DE3).

Expression, purification and refolding of pBV220-scFv

The expression of pBV220-scFv in *E. coli* BL21(DE3) was induced at OD_{600} ~0.6 by rising the temperature from 30 to 42 °C. Five hours after the induction, the cells were harvested by centrifugation (4200*g*, 10 min), suspended in buffer A (50 mM Tris–HCl, pH