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Expression, purification, and in vitro refolding of a humanized single-chain Fv antibody against human CTLA4 (CD152) ☆

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Abstract

A human-derived single-chain Fv (scFv) antibody fragment specific against human CTLA4 (CD152) was produced at high level in *Escherichia coli*. The scFv gene was cloned from a phagemid to the expression vector pQE30 with a N-terminal 6His tag fused in-frame, and expressed as a 29 kDa protein in *E. coli* as inclusion bodies. The inclusion body of scFv was isolated from *E. coli* lysate, solubilized in 8 M urea with 10 mM dithiothreitol, and purified by ion-exchange chromatography. Method for in vitro refolding of the scFv was established. The effects of refolding buffer composition, protein concentration and temperature on the refolding yield were investigated. The protein was renatured finally by dialyzing against 3 mM GSH, 1 mM GSSG, 150 mM NaCl, 1 M urea, and 50 mM Tris–Cl (pH 8.0) for 48 h at 4 °C, and then dialyzed against phosphate-buffered saline (pH 7.4) to remove remaining denaturant. This refolding protocol generated up to a 70% yield of soluble protein. Soluble scFv was characterized for its specific antigen-binding activity by indirect cellular ELISA. The refolded scFv was functionally active and was able to bind specifically to CTLA4 (CD152). The epitopes recognized by refolded anti-CTLA4 scFv do not coincide with those epitopes recognized by CD80/CD86.

Keywords: Single-chain Fv antibody; CTLA4; Bacterial expression; In vitro refolding

Cytotoxic T lymphocyte associated Ag-4 (CD152) is a structural homologue of the T cell co-stimulatory receptor CD28 and plays an inhibitory role in regulating T cell responses by interacting with the common ligands CD80/CD86 on APC [1,2]. The CTLA4's membrane expression is restricted to activated T cells and some tumor cells [3,4]. This Ag could be a good marker of T cell activation and a target for immunotherapy [5,6]. Engineered antibodies against CD152 have been used to study the structure and the role of the CD152 molecule, and to form immunotoxins [7]. Anti-CTLA4 single-chain Fv (scFv)[1] with the specific binding activity to CD152 can be potentially used in activated T cell and tumor cell targeting both in vitro and in vivo.

Single-chain Fv is a polypeptide consisting of the variable heavy chain (V_H) and the variable light chain (V_L), which are linked together by a 15- to 20-amino acid flexible linker, usually a $(Gly_4Ser)_3$ [8]. ScFv retains the binding specificity and affinity comparable to that of its parent antibody. Its small molecular size endows scFv with better tumor pene-

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[1] *Abbreviations used:* scFv, single-chain Fv; HAMA, human anti-mouse antibody; PCR, polymerase chain reaction; IPTG, isopropyl-D-thiogalactoside; DAB, 3,3′-diaminobenzidine; DTT, dithiothreitol; TMB, 3,3′,5,5′-tetramethylbenzidine.

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tration and lessened human anti-mouse antibody (HAMA) response. The scFv strategy has become one of the most popular methods in antibody engineering. And thus, scFv has a wide range of research, therapeutic, and diagnostic applications. A range of molecules have been fused with scFv to develop new functions, including biosensors for detection of target molecules [9], cytokines for immunotherapy [10], and radioisotopes for cancer imaging [11].

While mammalian cells secrete active scFvs, most single-chain antibodies tend to form inclusion bodies when expressed in bacteria, especially in *Escherichia coli*. However, high yield of scFv can be obtained from bacterial expression system allowing low-cost production, easy separation [12]. Therefore, establishing effective in vitro refolding method for scFv obtained from inclusion bodies would be very important.

In the present study, we constructed an expression vector pQEhS for anti-CTLA4 scFv, and transformed it to *E. coli* host strain M15. High level of expression was achieved in bacteria cells in the forms of inclusion bodies. After isolation and purification, an in vitro refolding method was established. The effects of refolding buffer composition, protein concentration, and temperature on the refolding yield were investigated. The antigen-binding property of the refolded protein was demonstrated by indirect cellular ELISA, and the antigen binding epitope of the scFv was analyzed by a competition binding experiment based on the indirect cellular ELISA. We obtained 30 mg of active scFv from 600 ml cultivation of *E. coli* cells. The refolded active scFv and the established method provide basis for further research in activated T cell and tumor cell targeting.

Materials and methods

Reagents

Platinum Pfx DNA polymerase was a product of Invitrogen Corporation. Restriction endonucleases, T4 DNA ligase, DNA marker DL2000, and X-gal were purchased from TaKaRa Biotechnology (Dalian, PR China). QIAprep Spin Miniprep Kit for plasmid extraction and RGS·His HRP Conjugate Kit for Western blot analysis were purchased from Qiagen. Prestained low range protein molecular weight marker was a product of MBI Fermentas. PVDF membrane was purchased from Bio-Rad. DEAE–Sepharose Fast Flow was a product of Amersham–Pharmacia Biotech. GSH and GSSG were purchased from Amresco. Human Burkitt's lymphoma cell line Raji and human umbilical vein endothelial cell lines ECV304 were stored in our laboratory. Anti-human CD152 mAb was purchased from Ancell. Other chemical reagents were of analytical grade.

Bacterial strains, media, and plasmids

The *E. coli* strain XL1-blue was stored in our laboratory, and was used as the host strain for cloning and maintenance of plasmids throughout the experiments. The *E. coli* strain M15 which was designed for regulated high-level protein expression was used as the host for expression vector in this study. The phagemid pBlueselect purchased from BBI was used for cloning and sequencing of the scFv gene. The plasmid pQE30 was used as an expression vector for expression of N-terminally hexahistidine-tagged protein. The *E. coli* strain M15 and plasmid pQE30 were purchased from Qiagen.

Construction of expression vector for scFv

The humanized anti-hCTLA4 scFv gene was amplified and modified using the polymerase chain reaction (PCR) with the phagemid pHEN1/anti-hCTLA4 scFv [13] as the template, and the following synthetic oligonucleotides, designed over the exact gene sequence, as primers: P1 (5′-CAGTGAGCTCATGGCCGAGGTGCAGCTGG-3′) and P2 (5′-CAGTGTCGACTCAACCTAGGACGGTCAGCTTGG-3′). These primers provided the amplified scFv gene with *Sac*I and *Sal*I restriction enzyme sites, respectively (underlined). Primer2 sequence contained some modifications required for the C-terminal stop codon. PCR was performed according to the method described in the manual of Platinum Pfx DNA Polymerase. Briefly, the PCR cycle was set at 94 °C for 45 s, 55 °C for 1 min, and 68 °C for 2 min for 30 cycles. PCR products were analyzed by 2% agarose gel electrophoresis and the expected size of the PCR product was cloned to pBlueselect and sequenced. The scFv gene with desired sequence was ligated to the *Sac*I and *Sal*I linearized vector pQE30, thus producing the expression vector for scFv. The inserted scFv gene was fused to N-terminal 6His-tag sequence in-frame.

Expression and Western blot analysis of scFv

For expression of the protein, the recombinant plasmid containing anti-hCTLA4 scFv was transformed by heat shock [14] into an *E. coli* host strain M15. Transformants were selected by growth on LB agar plates supplemented with 100 μg/ml ampicillin and 30 μg/ml kanamycin. Overnight colonies of transformants were cultured and induced with 0.1 mM isopropyl-D-thiogalactoside (IPTG) and allowed to grow for another 5 h at 37 °C. Aliquots of induced cells were disrupted by sonication. The total cell lysate before and after induction, soluble and insoluble fractions after sonication were analyzed by 12% SDS–PAGE carried out according to Laemmli [15] with a Mini Protean II apparatus (Bio-Rad). Protein gels were stained with Coomassie brilliant blue R250.

The SDS–PAGE gel was transferred to a PVDF membrane using Mini Trans-Blot cell (Bio-Rad) following manufacturer's instructions. The target protein was immunodetected by anti-His HRP conjugate following the instructions of QIAexpress Detection and Assay Handbook. Chromogenic-based detection was performed using 3,3′-diaminobenzidine (DAB) as a staining substrate.