Construction and Periplasmic Expression of a Bispecific Tandem scFv for Dual Targeting of Immune Checkpoints

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Abstract

Background: Immune checkpoints are molecules that act as regulators of immune system pathways. However, some tumor cells can express the ligands of immune checkpoints to escape from antitumor immune responses. Some agents, such as antibodies, can inhibit these checkpoints that prevent the immune system from targeting and killing cancer cells. The aim of this study was to express a novel bispecific tandem scFv in periplasmic space of *Escherichia coli* for simultaneous targeting of two immune checkpoints, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1).

Materials and Methods: The bispecific tandem scFv was constructed based on the variable regions gene of anti-PD1 and anti-CTLA-4 antibodies. The optimum codon for expression in *E. coli* was chemically synthesized and subcloned in periplasmic expression plasmid. After transformation, the effect of cultivation conditions on periplasmic expression of the protein in *E. coli* BL21(DE3) was evaluated. Then, the bispecific tandem scFv was purified and its binding ability to cells expressing PD-1 and CTLA-4 was evaluated.

Results: Expression of tandem scFv with a molecular weight of 55 kDa was verified by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting analysis. The best condition for soluble periplasmic expression was obtained to be incubation with 0.5 mM isopropyl β-D-1-thiogalactopyranoside at 23°C. The protein was successfully purified using affinity chromatography with a final yield of 4.5 mg/L. Binding analysis confirmed the bioactivity of purified the tandem scFv.

Conclusion: This bispecific tandem scFv could be a potential candidate to cancer immunotherapy, although more biological activity assessments are still required to be carried out.

Keywords: Antibodies, bispecific, CTLA-4, PD-1, neoplasms, periplasmic expression

INTRODUCTION

Immune checkpoint proteins including cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) are molecules that prevent damage to healthy cells in the body by inhibiting immune responses.[1,2] Some cancer cells can stimulate this process to protect themselves from an immune system attack. Immunosuppressive therapies are able to normalize immune function by blocking inhibitory immunosuppression. Some monoclonal antibodies (*e.g.,* nivolumab, ipilimumab) have been approved for clinical use based on targeting checkpoint proteins (*i.e.,* PD-1, PD-L1, or CTLA-4) on T cells to break T cell dysfunction, which increases the immune response against cancer cells.[3]

Recently, combination of nivolumab (*i.e.,* antibody targeting PD-1) and ipilimumab (*i.e.,* antibody targeting CTLA-4) was approved for first-line treatment of metastatic or recurrent nonsmall-cell lung carcinoma patients based on its synergistic

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effect on improvement of antitumor immune response.[4] Instead of using the combination of two immune checkpoint inhibitor antibodies, many researchers have been focusing on development of bispecific antibodies which simultaneously bind two immune checkpoint molecules.[5] Bispecific antibody is usually an engineered fusion protein consisting of two binding domains that simultaneously targets two antigens or epitopes.[6] There are several types of bispecific antibodies including hybrid IgGs, Fab fusion proteins, diabodies, and tandem scFvs.

scFvs formed from the connection of heavy and light chain variable domains (VH and VL) with a flexible peptide (*i.e.,* linker).[7] Bispecific tandem scFvs are made by connecting two different scFvs via a linker. These bispecific antibody formats, while maintaining their antigen binding properties, can be easily produced in bacterial cells; As the glycosylation of antibodies takes place in heavy constant domains, the production of tandem scFvs does not require glycosylation.[8]

Escherichia coli bacterial expression systems due to factors such as easy genetic manipulation, rapid growth, easy transformation, no need for complex culture media and high yield of expression are one of the best choices for the production of nonglycosylated recombinant proteins including tandem scFvs.[9] However, despite the benefits of produced proteins in *E. coli*, in general, the overexpression in cytoplasmic space of *E. coli* usually results in the formation of insoluble and biologically inactive protein aggregates (*i.e.,* inclusion bodies).[10] To prevent formation of inclusion bodies and to improve solubility of the proteins, different strategies have been applied including the secretion into periplasmic space, use of fusion proteins, mutations in the target protein, optimization of culture conditions and simultaneous expression with molecular chaperones.[11,12]

In this study, we reported periplasmic expression of a novel tandem scFv targeting CTLA-4 and PD-1. The effects of cultivation temperature and the inducer concentration on soluble expression of the target protein were investigated. After purification, the binding ability of the protein was evaluated by flow cytometry.

MATERIALS AND METHODS

Design and construction of the bispecific tandem scFv gene

The bispecific tandem scFv antibody was designed according to the amino acid sequences of the variable light and heavy chains of nivolumab and ipilimumab joined together by a flexible linker. The designed sequence was codon optimized for its expression in *E. coli*. The sequence also contained a carboxy-terminus 6 × His-tag and a stop codon. The gene was synthesized by Biomatik Company (Canada) and subcloned into the *Nco*I and *Xho*I restriction sites of pET-22b expression vector (Novagen, USA) in frame with the pelB signal sequence.

Expression of the bispecific tandem scFv

E. coli BL21 (DE3) was transformed with the recombinant pET22b vector using the CaCl$_2$ method.[13] A positive colony was inoculated to a sterile tube containing Luria–Bertani (LB) broth supplemented with 100 µg/mL ampicillin and incubated at 37°C overnight. The next day, the culture was inoculated into the fresh medium with a ratio of 1:100 and incubated at same condition until OD$_{600}$ reached 0.5. Then, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), as a protein expression inducer, was added to the culture and incubated at 37°C for 3 h. Finally, the culture was centrifuged at 7,500 *g* for 10 min, and the supernatant was discarded by aspiration. The cell pellet was stored at −70°C for further analysis.

Effect of cultivation conditions on periplasmic soluble expression of the bispecific tandem scFv

The overnight culture of *E. coli* BL21 (DE3) containing the recombinant pET22b vector was used to find optimum temperature and IPTG concentration for the periplasmic soluble expression of the bispecific tandem scFv. The culture was inoculated to fresh LB and incubated at 37°C until reaching the logarithmic growth phase. Next, the culture was incubated with different inducer concentrations (0.25, 0.5, and 1 mM) at 37°C, 30°C, and 23°C for 3, 4.5, and 18 h, respectively.

Periplasmic isolation and purification of the bispecific tandem scFv

After washing with phosphate buffer saline buffer, the pellet was dispersed in a cold hypertonic buffer (500 mM sucrose, 1 mM ethylene diamine tetra acetic acid, 0.05 M Tris-HCl, pH 8.0) and incubated at 100 rpm at 4°C for 45 min. To separate the hypertonic fraction, the sample was subjected to centrifugation at 9,000 g for 10 min at 4°C. Then, the pellets were resuspended in a cold hypotonic buffer (5 mM MgSO$_4$) and incubated at 100 rpm at 4°C for 30 min. The resulting suspension was centrifuged at the same condition and the supernatant was harvested as the hypotonic extract.

Hypertonic and hypotonic fractions were pooled and dialyzed against the binding buffer (0.05 M monosodium phosphate and 0.5 M NaCl, pH 8.0). Then, the sample was loaded into an affinity column containing Ni-NTA agarose resin (Invitrogen, USA) and gently agitated for 45 min at room temperature. After four times washing the column with the wash buffer (0.05 M monosodium phosphate, 0.5 M NaCl and 20 mM imidazole, pH 8.0), the bispecific tandem scFv was eluted using the elution buffer (0.05 M monosodium phosphate, 0.5 M NaCl and 50–250 mM imidazole, pH 8.0).

Analytical methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed to analyze the protein samples. The Bradford method was used to determine the purified tandem scFv antibody concentration.[14] The final yield of soluble protein production for 1 L of bacterial culture was calculated using the following equation where 10 is the concentration factor used

in the preparation of total protein lysate samples and 5 is the volume (mL) of eluted purified samples: (mg/L) = concentration of protein (mg/mL) × 10 × 5. To evaluate the biological activity of purified tandem scFv, binding assays were performed by flow cytometry. Briefly, $5 × 10^5$ ConA stimulated peripheral blood mononuclear cells (PBMCs) (expressing PD-1 and CTLA-4) were incubated with tandem scFv or isotype control antibody (anti-HER2 scFv containing a carboxy-terminus 6 × His-tag). After three times washing, cells were treated with the secondary antibody (anti-His-FITC). Then cells were washed and analyzed using a flow cytometer (BD, USA).

RESULTS

Expression of the bispecific tandem scFv

Our results suggested that the bispecific tandem scFv could be successfully expressed under general induction conditions (i.e., 1 mM IPTG at 37°C) in *E. coli* BL21 (DE3). Three hours after the addition of inducer, the produced tandem scFv represented approximately 25% of total protein content [Figure 1a]. The expected molecular weight of the full-length his-tagged tandem scFv was about 55 kDa, which was proved by SDS-PAGE analysis [Figure 1a] and western blotting analysis [Figure 1b].

Optimization of the bispecific tandem scFv expression

To assess the effects of different inducer concentrations and cultivation temperatures on periplasmic expression of tandem scFv, different conditions were evaluated. SDS-PAGE analysis showed when the induction temperature reduced from 37°C to 23°C, soluble periplasmic expression of protein remarkably improved [Figure 2]. Our finding indicated higher soluble periplasmic tandem scFv was produced at 0.5–1 mM IPTG. Accordingly, the maximum expression of protein was achieved with 0.5 mM IPTG at 23°C.
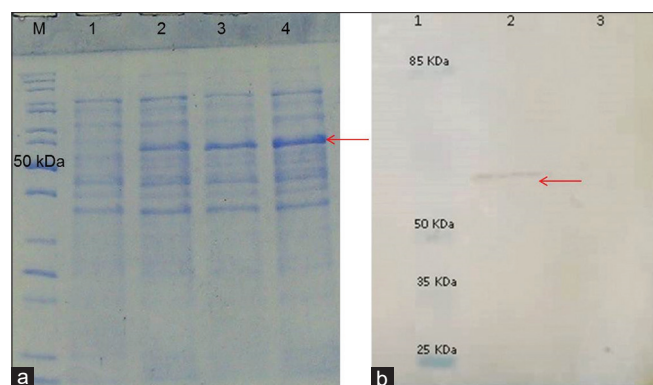

Figure 1: (a) SDS-PAGE analysis of protein expression in *E. coli* BL21 (DE3) harboring pET22b-tandem scFv vector. Lanes 1 to 4 show protein expression with 1 mM IPTG at 37°C at different times, 0, 1, 2, and 3 h, respectively. Lane M: protein marker. (b) Western blot analysis of protein expression in *E. coli* BL21 (DE3) harboring pET22b-tandem scFv vector with anti-His-HRP antibody. Lane 1: protein marker; Periplasmic fractions after (lane 2) and before (lane 3) induction with 1 mM IPTG for 3 h at 23°C. Tandem scFv (55 kDa) is showed by arrows

Purification and bioassay of the bispecific tandem scFv

The soluble bispecific tandem scFv was successfully purified using the native Ni NTA affinity column. SDS-PAGE analysis revealed that the native purification led to about 95% purity and a final yield of 4.5 mg/L [Figure 3]. Figure 4 shows the results of flow cytometric analysis. The binding ability of the bispecific tandem scFv was quantified by the following equation: [geometric mean fluorescence intensity (MFI) of treated cells/geometric MFI of untreated cells] × 100. Our data showed that the ratio of geometrical MFI of treated relative to nontreated cells was 1.44.

DISCUSSION

Dual-specific antibody (*i.e.,* bispecific antibody) is an alternative solution for simultaneous use of two antibodies therapy.[15] So far, two bispecific antibodies (*i.e.,* blinatumomab and catumaxomab) have been approved by the FDA and more than 30 antibodies are in clinical trial stages.[16] Blinatumomab is a tandem scFv produced by the bispecific T cell engager technology[17] that simultaneously targets CD3 on T and CD19 on B cells. This bispecific antibody is used to treat acute lymphoblastic leukemia in children and adults.[18]

In the case of simultaneous targeting of PD-1 and CTLA-4 receptors, several bispecific antibody formats have been proposed, some of which are going through clinical trials. For example, XmAb20717 or vudalimab is a bispecific PD-1/CTLA-4 antibody[19] that the Fc section is engineered to improve efficacy (*e.g.,* better antibody functions such as Antibody-dependent cellular cytotoxicity) and to enhance the stability and half-life of the antibody. Preliminary data of phase I showed that the antibody is safe and effective in patients with advanced solid tumors.[20]

Here, we developed a novel bispecific tandem scFv for co-targeting PD-1 and CTLA-4. Unlike IgG-like formats, tandem scFvs exhibit better tissue penetration due to their smaller size.[21] Furthermore, they can be easily expressed in prokaryotic cells like bacteria. *E. coli* has been widely applied as a host cells for expression of recombinant proteins. However, production of a functionally bioactive and correctly folded protein is the main challenge of this expression platform.

The periplasmic expression has some advantages over cytoplasmic platform including oxidizing environment of periplasmic space and the presence of some chaperons (*e.g.,* DsbA and DsbC) which facilitate formation of correct disulfide bonds, and lower level of proteases and low number of contaminate proteins which help easier downstream processing compared with cytoplasmic expression.[22] However, this platform usually led to lower yield of the protein. To improve the yield and recovery of proteins different cultivation, recovery and purification stages must be optimized.

The optimum soluble expression of the bispecific tandem scFv was achieved by adjusting the cultivation temperature and the inducer concentrations.
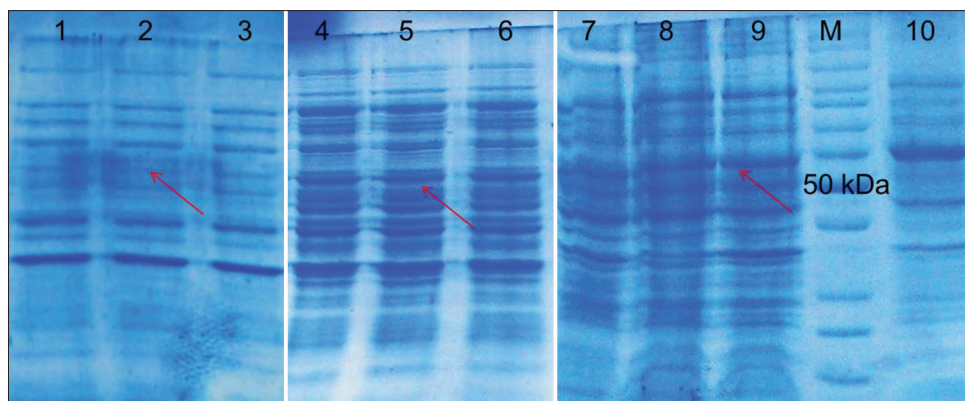
Figure 2: SDS-PAGE analysis of soluble periplasmic expression of tandem scFv. Periplasmic fractions of *E. coli* BL21 (DE3) harboring pET22b-tandem scFv vector after induction of protein expression with 0.25 mM (lane 1, lane 4, and lane 7), 0.5 mM (lane 2, lane 5, and lane 8) and 1 mM (lane 3, lane 6, and lane 9) IPTG at 37°C, 30°C, and 23°C, respectively. Lane M: Protein marker; lane 10: total protein after expression with 1 mM IPTG at 37°C. Tandem scFv (55 kDa) is showed by arrows
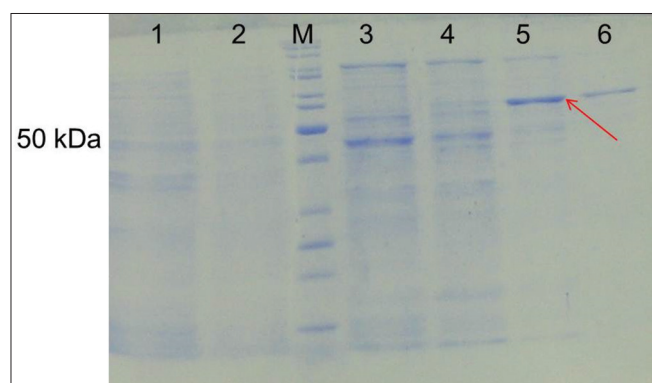

Figure 3: SDS-PAGE analysis of the purified tandem scFv using a Ni-NTA column under native conditions. Lane 1: wash 1; lane 2: wash 2; lane M; protein marker; lane 3: flow-through after loading onto the column; lane 4: periplasmic fraction before loading onto column; lane 5: elute 1 (50 mM imidazole); line 6: elute 2 (100 mM imidazole). Tandem scFv (55 kDa) is showed by arrows
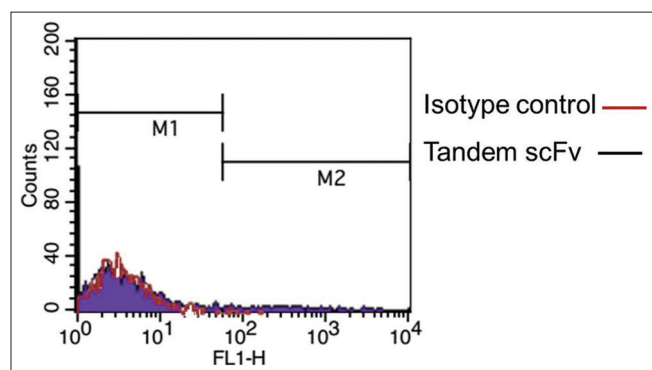

Figure 4: Flow cytometric analysis. The binding ability of tandem scFv (black line) to PBMS cells was evaluated by flow cytometry. The isotype antibody was considered a negative control (red line)

The inducer concentrations did not significantly influence the soluble expression of the protein in the range of 0.25–1 mM of IPTG. Similarly, another work reported no significant difference in the expression of anti-EGFRvIII scFv in the periplasm and cytoplasm with IPTG concentration from 0.1 to 1 mM.[23] However, lower concentrations of IPTG (0.01–0.1 mM) resulted in 2 to 10-fold increase in periplasmic expression of a bioactive Fab fragment.[24] It suggested that higher inducer concentration led to higher expression rates resulting in more insoluble and aggregated proteins.[24]

In this study, lowering the culture temperature to 23°C led to improved soluble periplasmic expression of the protein. Other studies also reported that reducing the cultivation temperature led to significant improvement in soluble expression of recombinant antibody fragments due to reduction of the rate of protein synthesis that facilies the correct folding of the protein and prevent aggregate formation, as well as reduction of protein degradation.[25,26] For example, it was previously reported that the soluble periplasmic expression of the Fab fragment of the human monoclonal antibody 3H6 was improved by decreasing cultivation temperature below 30°C.[25]

The yield of tandem scFv recovered and purified from periplasmic space of *E. coli* was 4.5 mg per 1 L of bacterial culture. We previously expressed and purified periplasmic anti-HER2 scFv using Ni-NTA-agarose affinity chromatography under native conditions which yielded 2.7 mg/g of wet cells.[13] In another study, after periplasmic expression of a bispecific antibody, HER2/CD3-S-Fab, the protein was recovered from periplasmic fraction or M9 minimal medium using Ni-NTA-agarose affinity chromatography. They found that M9 medium could facilitate the secretion of the protein into the medium culture and increased the yield of HER2/CD3-S-Fab up to 3 mg per L of the culture.[27]

Flow cytometric analysis confirmed the biological activity of purified tandem scFv based on its binding to cell expression PD-1 and CTLA-4. For binding assay, we used ConA stimulated PBMCs. Previous studies showed that ConA stimulation up-regulation of CTLA-1 and PD-1 in T cells, in the presence of antigen-presenting cells, and PBMCs.[28,29]

Conclusion

In this study, we reported periplasmic expression of a bispecific tandem scFv against PD-1 and CTLA-4. The expression of protein was verified by SDS-PAGE and western blot. The best condition for periplasmic expression of soluble protein was incubation with 0.5 mM IPTG at 23°C which after purification led to a final yield of 4.5 mg/L. The binding analysis confirmed the functional expression and purification of the tandem scFv. Development of novel immune therapeutic agents like our tandem scFv will benefit the advancement of cancer research. However, more *in vitro* and *in vivo* biological activity assessments are still required to be carried out.

Ethical Approval

This study was approved by Research Ethics Committees of Vice-Chancellor in Research Affairs -Medical University of Isfahan. (Ethical approval code: IR.MUI.RESEARCH. REC.1398.577)

Conflicts of interest

There are no conflicts of interest.

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