The Construction of the Four Recombinant Antibody Plasmids from the Two Anti-EGFR Human Single-domain Antibodies and Their Expression

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Abstract

Monoclonal antibodies (mAbs) for cancer therapy have some limitations. It is important to search for small-size antibodies including single-domain antibodies (sdAbs) to replace mAbs for cancer therapy. Two anti-EGFR sdAbs (pET22b-aEG2E12 and pET22b-aEG4D9) were previously isolated in our laboratory and showed the in vitro and in vivo anti-tumor activity. In this study, the four anti-EGFR recombinant antibody plasmids were constructed from these two sdAbs. They include the bivalent recombinant plasmids pET22b-aEG4D9-aEG2E12 and pcDNA3.1-aEG4D9-aEG4D9. The recombinant antibody plasmid pET22b-aEG4D9-aEG2E12-PE was constructed by combining the bivalent antibody pET22b-aEG4D9-aEG2E12 and PE38 (the 38 kDa fragment of Pseudomonas exotoxin A). The plasmid pcDNA3.1-aEG4D9-anti-CD3-aEG4D9 was constructed by combining the bivalent antibody pcDNA3.1-aEG4D9-aEG4D9 and anti-CD3 single-chain variable fragment (scFv). SDS-PAGE gel electrophoresis was performed to detect the expression of the four anti-EGFR recombinant antibodies. Two recombinant antibody proteins (pET22b-aEG4D9-aEG2E12 and pET22b-aEG4D9-aEG2E12-PE) were obtained by the expression and purification of bacteria soluble proteins. Two recombinant antibody proteins (pcDNA3.1-aEG4D9-aEG4D9 and pcDNA3.1-aEG4D9-anti-CD3-aEG4D9) were obtained by expression of eukaryotic cells 293f. This study can lay a necessary foundation for the further study of the in vitro and in vivo functions of these four anti-EGFR recombinant antibodies and their use for cancer therapy.

Keywords

EGFR; Single-domain Antibody; PE; Anti-CD3 Single-chain Variable Fragment; Recombinant Protein.

1. Introduction

EGFR is a 170 kDa transmembrane protein, belonging to the type I growth factor receptor family (ErbB/HER receptor family) of receptor tyrosine kinases (RTKs) [1]. In many adult tissues, EGFR is normally expressed and participates in cellular processes such as cell survival and cell growth [2]. Recent studies have found that EGFR is abnormally overexpressed in a variety of cancers and is associated with poor prognosis [3]. The mechanism is that after the over-expressed EGFR binds to its ligand EGF in tumor cells, EGFR is activated, leading to the activation of three downstream signaling pathways (including the RAS-RAF-MEK-ERK pathway, the JAK-STAT pathway and the PI3K-AKT pathway), thereby promoting tumor cell proliferation and invasion and inhibiting apoptosis [4-5]. Therefore, EGFR has become a good target for cancer therapy.

Currently, there are two classes of anti-cancer drugs against EGFR: tyrosine kinase inhibitors and monoclonal antibodies [6]. FDA has approved Cetuximab and Panitumumab for the clinical treatment of metastatic colorectal cancer, both of which are monoclonal antibodies (mAbs) against EGFR [7]. However, there are still many problems in the use of mAbs for cancer therapy, such as: high molecular weight (about 150 kDa), making it difficult to penetrate into solid tumors [8]. Murine mAbs are

immunogenic [9], and the production of mAbs by mammalian cells is expensive [10]. Therefore, it is necessary to search for small-size antibodies to replace murine mAbs for cancer therapy.

Single-domain antibody (sdAb) is the smallest complete antigen binding fragment with a molecular weight of only 12–15 kDa [11]. sdAb has high stability, high solubility, high affinity and better tissue permeability, which provides the broad applications in tumor treatment and diagnosis [12]. Two anti-EGFR human sdAbs (pET22b-aEG2E12 and pET22b-aEG4D9) were previously isolated in our laboratory and expressed in bacteria [13]. It was confirmed that both had anti-tumor activity in vitro and in vivo functional assays [13]. Due to the lack of Fc domain, sdAbs lack immune cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Therefore, sdAbs need to be changed for better therapeutic effect by the methods including the construction of bivalent sdAbs and bispecific sdAbs and the use of sdAbs to deliver toxins, drugs, radionuclides and peptides [14].

Bivalent sdAbs are formed by fusion of two sdAbs targeting the same antigen by tandem cloning or by a polypeptide linker. Some studies showed that bivalent antibodies had better therapeutic effect, including higher affinity, longer clearance time and more functions [15]. Pseudomonas exotoxin A (PE) is an effective cell-killing agent, the toxin can be linked with the sdAb to form the recombinant protein. The recombinant protein uses the sdAb to target the related antigen on the surface of cancer cell, and PE passes through the cellular membrane and binds to the ADP-ribosylation elongation factor II, thereby preventing the protein synthesis and inducing cell apoptosis [16-17]. The bispecific antibody (CD3-BsAb) against CD3 antigen proved to have good clinical potential [18]. It targets both the CD3 domain on the surface of T cells and the abnormally expressed antigen on the surface of tumor cells. The two kinds of cells can form immune synapses through the cross-linking of CD3-BsAb, which can induce T cells to activate, secrete inflammatory cytokines (including IFN-γ, TNF-α, IL-2 and IL-10, etc.) and cytolytic molecules and induce tumor cell apoptosis [19]. In this study, the four anti-EGFR recombinant antibodies were constructed from the two anti-EGFR sdAbs (pET22b-aEG2E12 and pET22b-aEG4D9) isolated previously in our laboratory [13]. SDS-PAGE gel electrophoresis was performed to determine the expression and purification of the four anti-EGFR recombinant antibodies. This study can lay a necessary foundation for further study of the anti-tumor effects of the four anti-EGFR recombinant antibodies.

2. Materials and methods

2.1 Reagents and cell culture

Isopropyl-β-D-thiogalactopyranoside (IPTG) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sangon Biotech (Shanghai, China). Nickel nitrilotriacetic acid (Ni-NTA) resin was purchased from Sevensea Biotech (Shanghai, China). KOP293, KPM, TA-293 and KE-293 were purchased from Kairui Biotech (Zhuhai, China). Mini plasmid kit and endo-free plasmid maxi kit were purchased from Omega Biotek Inc (Doraville, GA, USA).

The 293f cell line were provided by Kairui Biotech (Zhuhai, China). 293f cells and cell culture medium (KOP293) were added to a sterile Erlenmeyer flask, and cultured in a constant temperature oscillation incubator at 37°C, 5% CO_2, and 110 rpm.

2.2 Construction of the prokaryotic and eukaryotic expression plasmids

The bivalent sdAb plasmid pET22b-aEG4D9-aEG2E12 was constructed by linking the two sdAbs (aEG2E12 and aEG4D9) with $(G_4S)_3$ linker and using pET-22b plasmid, and the 3' end of the bivalent antibody was linked with PE38 through $(G_4S)_3$ linker to form a recombinant antibody plasmid pET22b-aEG4D9-aEG2E12-PE. In addition, the eukaryotic expression plasmid pcDNA3.1-aEG4D9 and its bivalent sdAb plasmid pcDNA3.1-aEG4D9-aEG4D9 were constructed. This bivalent antibody and the anti-CD3 scFv were ligated by (G_4S) linker to form a recombinant antibody plasmid pcDNA3.1-aEG4D9-anti-CD3-aEG4D9. The construction of the plasmids above were completed by Jinweizhi Company.

2.3 Expression and purification of the four anti-EGFR recombinant antibody proteins

The prokaryotic expression plasmids were extracted according to the instructions of the mini plasmid kit (Omega Biotek Inc). The plasmids were transformed into E. coli BL21 (DE3) (Novagen). A single clone was randomly picked and incubated in LB medium containing ampicillin at 220 rpm at 37℃ overnight. IPTG (Sangon Biotech) was added, and the bacteria were cultured at 22℃ for 6 h to induce protein expression. The harvested culture medium was centrifuged, and the bacteria precipitates were resuspended in PBS containing PMSF (Sangon Biotech). After ultrasonically breaking down the bacteria and centrifugation, the supernatant containing the soluble protein extract was obtained. The supernatant was added to the Ni-NTA resin column (Sevensea Biotech) to purify the protein. Proteins were examined by 15% SDS-PAGE and Nanodrop was used to measure protein concentrations.

The eukaryotic expression plasmids were extracted according to the instructions of the endo-free plasmid maxi kit (Omega Biotek Inc). The trasfection was performed according to the manufacture's protocol (Kairui Biotech). KPM (Kairui Biotech) and plasmids were added to centrifuge tube 1, and KPM and TA-293 (Kairui Biotech) were added to tube 2. The liquid from tube 2 was added to tube 1. The plasmid-vector complex was prepared by incubating at room temperature for 10 min. The 293f cells (Kairui Biotech) were taken out from the constant temperature oscillation incubator, and the plasmid-vector complex was added to the cells. After culturing for 24 h, KE-293 (Kairui Biotech) was added. The supernatant was collected by centrifugation, and the protein was purified by Ni-NTA resin column (Sevensea Biotech) after 6 days of culture. The proteins were examined by 15% SDS-PAGE, and the protein concentrations were measured using Nanodrop.

3. Results

3.1 Construction of the four anti-EGFR recombinant antibody plasmids

DNA sequences of the two sdAbs (aEG2E12 and aEG4D9) were ligated by adding the $(G_4S)_3$ linker, and then, the ligated product was inserted between Nco I and Not I sites of pET-22b plasmid to construct the recombinant plasmid pET22b-aEG4D9-aEG2E12. Using the same linker, the DNA sequence of PE38 was added to the 3' end of the ligated product of the bivalent sdAbs, and the recombinant antibody plasmid pET22b-aEG4D9-aEG2E12-PE was constructed.

DNA sequence of the sdAb (aEG4D9) was inserted between EcoR V and Not I sites of pcDNA3.1/Myc(+)/His(+) plasmid to construct plasmid pcDNA3.1-aEG4D9. The bivalent sdAb plasmid pcDNA3.1-aEG4D9-aEG4D9 was constructed by using the $(G_4S)_3$ linker and also inserting between EcoR V and Not I sites of pcDNA3.1/Myc(+)/His(+) plasmid. Using the (G_4S) linker, the DNA sequence of anti-CD3 scFv was inserted into pcDNA3.1-aEG4D9-aEG4D9 to construct the recombinant antibody plasmid pcDNA3.1-aEG4D9-anti-CD3-aEG4D9.

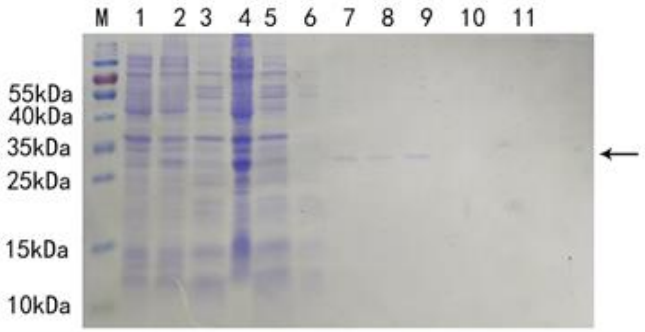

Fig. 1 The expression and purification of pET22b-aEG4D9-aEG2E12 was determined by SDS-PAGE. Lane M, protein marker; Lane 1, total proteins of the un-induced bacteria; Lane 2, total protein of induced bacteria; Lane 3, soluble supernatant after bacteria lysis; Lane 4, insoluble portion after bacteria lysis; Lane 5, flow-through fraction; Lane 6, wash buffer; Lane 7-11, the recombinant proteins collected from the purification column. The position of the protein band was marked with the arrow.

3.2 Expression and purification of pET22b-aEG4D9-aEG2E12

The plasmid pET22b-aEG4D9-aEG2E12 was transformed into E. Coli BL21 (DE3), and the bacteria culture was spread on a plate. A single clone was randomly picked from the plate and was cultured. IPTG was added at a final concentration of 0.5 mM to induce soluble protein expression at 25°C. After 6 h of culture, the bacteria were broken down, the supernatant was collected and purified with a Ni-NTA resin column. Finally, the recombinant proteins were obtained through dialysis and concentration. SDS-PAGE gel electrophoresis was performed to determine the protein expression of pET22b-aEG4D9-aEG2E12. As shown in Fig. 1, the molecular weight of pET22b-aEG4D9-aEG2E12 protein is about 30kDa, and the protein location was marked with the arrow. Ater purification by the Ni-NTA resin column, the eluted protein was a single band. The concentration of the recombinant protein was determined by Nanodrop, and the protein yield was between 0.7-0.9 mg in 1 L bacteria culture.

3.3 Expression and purification of pET22b-aEG4D9-aEG2E12-PE

The expression plasmid pET22b-aEG4D9-aEG2E12-PE was transformed into E. coli BL21 (DE3). A single clone was randomly picked and cultured, and IPTG was added at a final concentration of 0.5 mM to induce protein expression at low temperature. A Ni-NTA resin column was used to purify the recombinant proteins from the supernatant after bacteria breakage, and further dialysis was done to obtain the pure proteins. SDS-PAGE gel electrophoresis was performed to determine the expression of pET22b-aEG4D9-aEG2E12-PE protein. As shown in Fig. 2, the molecular weight of the recombinant protein is about 70 kDa, and its position was marked by the arrow. After purification by the Ni-NTA resin column, the eluted protein showed a single band. The recombinant protein concentration was determined by Nanodrop, and its yield was between 0.7-0.9 mg in 1 L bacteria culture.

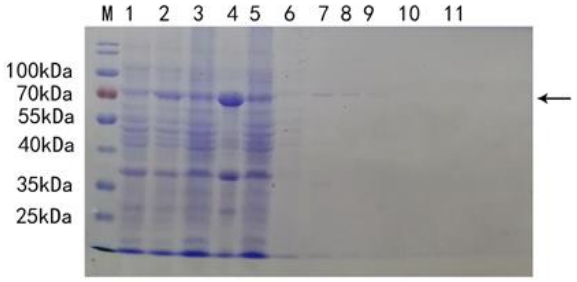

Fig. 2 SDS-PAGE was performed to detect the expression and purification of pET22b-aEG4D9-aEG2E12-PE. Lane M, protein marker; Lane 1, total protein of the un-induced bacteria; Lane 2, total protein of induced bacteria; Lane 3, soluble supernatant after bacteria lysis; Lane 4, insoluble portion after bacteria lysis; Lane 5, flow-through fraction; Lane 6, wash buffer; Lane 7-11, the recombinant protein collected from the purification column. The position of the protein band was marked with the arrow.

3.4 Expression and purification of pcDNA3.1-aEG4D9-aEG4D9

The plasmid pcDNA3.1-aEG4D9-aEG4D9 was extracted according to the instruction of endo-free plasmid maxi kit. Then, the expression plasmid was transfected into 293f cells at a density of 3×10^6 cells/ml, and KE-293 (protein expression enhancer) was added 24 h after transfection. The transfection culture was incubated for 6 days, the cell culture supernatant was purified using Ni-NTA resin column, and the pure recombinant protein was obtained by dialysis. The proteins obtained during the transfection were analysed by SDS-PAGE gel electrophoresis in order to verify the expression of the target protein. As shown in Fig. 3, the molecular weight of pcDNA3.1-aEG4D9-aEG4D9 is about 40 kDa, and its position was marked by the arrow. After purification by the Ni-NTA resin column, the eluted protein showed a single band. The concentration of the recombinant protein was determined by Nanodrop, and the protein yield was between 8-10 mg in 1 L cell culture medium.

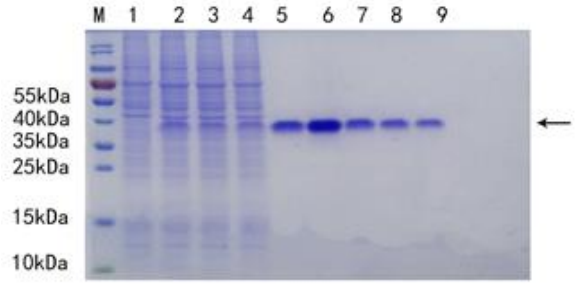

Fig. 3 The expression and purification of pcDNA3.1-aEG4D9-aEG4D9 was determined by SDS-PAGE. Lane M, protein marker; Lane 1, supernatant of the un-transfected cells; Lane 2, supernatant of the transfected cells; Lane 3, flow-through fraction; Lane 4, wash buffer; Lane 5-9, the recombinant proteins collected from the purification column. The position of the protein band was marked with the arrow.

3.5 Expression and purification of pcDNA3.1-aEG4D9-anti-CD3-aEG4D9

The plasmid pcDNA3.1-aEG4D9-anti-CD3-aEG4D9 was extracted according to the instruction of endo-free plasmid maxi kit. Then, the recombinant plasmid was transfected into 293f cells (at a density of 3×10^6 cells/ml and viability greater than 95%), and KE-293 was added 24 h later. After 6 days of culture, the transfection was finished. The cell culture supernatant was collected and purified with the Ni-NTA resin column, and the pure protein could be obtained by dialysis. Samples collected during the transfection were analysed by SDS-PAGE gel electrophoresis in order to verify the expression of pcDNA3.1-aEG4D9-anti-CD3-aEG4D9. As shown in Fig. 4, the molecular weight of the recombinant protein is about 70 kDa, and its position was marked by the arrow. After the cell culture supernatant was purified by the Ni-NTA resin column, the eluted protein was a single band. The recombinant protein concentration was determined by Nanodrop, and its yield was between 8-10 mg in 1 L cell culture medium.

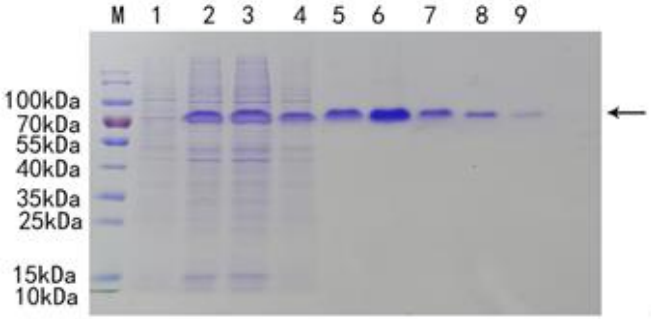

Fig. 4 SDS-PAGE was performed to detect the expression and purification of pcDNA3.1-aEG4D9-anti-CD3-aEG4D9. Lane M, protein marker; Lane 1, supernatant of the un-transfected cells; Lane 2, supernatant of the transfected cells; Lane 3, flow-through fraction; Lane 4, wash buffer; Lane 5-9, the recombinant protein collected from the purification column. The position of the protein band was marked with the arrow.

4. Discussion

EGFR is a receptor tyrosine kinase that can regulate cell growth and cell differentiation. Its abnormal activation and overexpression were confirmed to be related to the occurrence of many human malignancies and is a good target for cancer therapy. Two sdAbs against EGFR was previously isolated in our laboratory, and their anti-tumor activity was confirmed [13]. In order to increase the anti-tumor effect, these antibodies need to be changed. Shimazaki et al found that in the murine models the bivalent antibody could undergo rapid tumor uptake, showing its potential for cancer therapy [20]; Bispecific antibody is an emerging type of tumor-targeting immunomodulator, which

can further improve the clinical efficacy and safety [21]. Hou et al showed that scFv could be conjugated with PE38, and in comparison with a single scFv, the bivalent antibody could better promote the delivery of toxins, resulting in stronger cellular toxicity [22]. Reusch et al coupled anti-CD3 antibody with specific antibody targeting tumor cell surface antigen to generate effective immune synaptic conformation between T cells and target cells, trigger T cell activation and proliferation and induce tumor cell apoptosis [23]. In this study, two bivalent sdAb plasmids pET22b-aEG4D9-aEG2E12 and pcDNA3.1-aEG4D9-aEG4D9 were constructed. In addition, pET22b-aEG4D9-aEG2E12 was combined with PE38 to construct an anti-EGFR recombinant antibody plasmid pET22b-aEG4D9-aEG2E12-PE. The pcDNA3.1-aEG4D9-aEG4D9 was combined with anti-CD3 scFv to construct a recombinant antibody plasmid pcDNA3.1-aEG4D9-anti-CD3-aEG4D9. SDS-PAGE gel electrophoresis showed that the two anti-EGFR recombinant antibody proteins pET22b-aEG4D9-aEG2E12 and pET22b-aEG4D9-aEG2E12-PE could be expressed and purified by prokaryotic bacteria, and their yields were between 0.7-0.9 mg in 1 L bacteria culture. The two recombinant antibodies pcDNA3.1-aEG4D9-aEG4D9 and pcDNA3.1-aEG4D9-anti-CD3-aEG4D9 could be expressed by eukaryotic cells 293f, and their yields were between 8-10 mg in 1 L cell culture medium. This study showed that the four anti-EGFR recombinant antibodies could be expressed and purified, laying the necessary foundation for their subsequent anti-tumor research.

5. Conclusion

Two sdAbs were changed to obtain four anti-EGFR recombinant antibodies. pET22b-aEG4D9-aEG2E12 and pET22b-aEG4D9-aEG2E12-PE could be expressed and purified in prokaryotic bacteria, while pcDNA3.1-aEG4D9-aEG4D9 and pcDNA3.1-aEG4D9-anti-CD3-aEG4D9 could be expressed and purified in eukaryotic cells 293f. Our study can lay a necessary foundation for further study of the anti-tumor effects of these four anti-EGFR recombinant antibodies.

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