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Original article

Prokaryotic expression and refolding of EGFR extracellular domain and generation of phage display human scFv against EGFR

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ABSTRACT

The epidermal growth factor receptor (EGFR), overexpressed in many epithelial tumors, is emerging as an attractive target for cancer therapy. Antibodies to the extracellular region of EGFR play a key role in the development of a mechanistic understanding and cancer therapy. In the present study, we demonstrated for the first time that EGFR-truncated extracellular domain (EGFR-tED), which was expressed in *Escherichia coli* BL21 (DE3) cells in the form of inclusion bodies, could be purified and renatured. The EGFR-tED protein was purified by gel filtration and Ni-NTA affinity chromatography with high purity ($>$ 90%) and refolded by a urea gradient size-exclusion chromatography, which could bind its ligand EGF in a concentration-dependent manner. The renatured EGFR was used for biopanning anti-EGFR scFvs from a human synthetic antibody phage display library. Combined with an additional cell-based ELISA screen, a novel scFv, E10, was obtained with two-fold more potent on the binding to EGFR-bearing tumor cells (the epidermoid carcinoma cell line A431) and the inhibition of A431 cells proliferation than scFv 11F8, suggesting that the E10 has the potential to be developed as therapeutic agents to solid tumors associated with EGFR overexpression.

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1. Introduction

Epidermal growth factor receptor (EGFR or HER) family comprises of four distinct transmembrane receptors: EGFR or HER1/erbB1, HER2/erbB2, HER3/erbB3, and HER4/erbB4 [1]. The first molecularly cloned EGFR is a large (1186 residues), modular glycoprotein with an extracellular ligand-binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain [2]. EGFR is overexpressed in a large number of human tumors, including carcinomas of the head and neck, breast, colon, prostate, lung, ovaries and sinonasal squamous cell [3,4]. Overexpression of EGFR is correlated with an unfavorable prognosis, altered response to chemotherapy, and decreased survival [2,5], It is also frequently accompanied by the production of its natural ligands, mainly EGF or TGF-α by tumor cells, suggesting that an autocrine loop participates in malignant transformation [3], which makes it a significant therapeutic target for cancer [6,7].

Two main classes of compounds that have been currently developed targeting EGFR are the small-molecule inhibitors of the intracellular tyrosine kinase domain and monoclonal antibodies (mAbs) directed against the extracellular region of the receptor. Even though a few anti-EGFR mAbs are currently in clinic or clinical trials, they exhibit varying properties [8], For example, cetuximab and panitumumab were recently marketed for colon, head and neck, and/or lung cancers, covering limited ranges of solid tumors. Phase II study of IMC-11F8 [9,10] in patients with colorectal cancer has been completed (NCT00835185). Considering the diversity of the EGFR-associated solid tumors, it is necessary to develop more anti-EGFR antibodies to be applied to other solid tumors [6,8,11].

EGFR have been expressed or extracted from different eukaryotic hosts for the structure-based drug design and screen or preparation of its antibodies, such as HEK293 cell line, rat astrocytes, breast cancer cell line SKBR3 and Chinese hamster ovary cells [12]. The extracellular portion of EGFR is divided into four domains (I–IV). Domain I and III of the receptor have been shown to play minor and major roles, respectively, in the ligand binding, while domain II mediates receptor dimerization [13]. A truncated EGFR extracellular domain (1–501 residues, including domain I to III and the first module of domain IV) expressed in HEK293 cell line, binds human EGF or TGFα with 13-fold higher affinity than the full-length EGFR ectodomain [14,15].

However, prokaryotic hosts, e.g. *Escherichia coli (E. coli)*, has not been reported before to obtain this kind of recombinant molecules mainly for the formation of inclusion bodies, which are hard to

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refold because of the misfolding and aggregation caused by its cys-rich domains. Nevertheless, due to the limitations of eukaryotic expression systems, such as low yield of recombinant protein, high cost, complex to construct and time-consuming for expression, strategies of solubilizing and refolding recombinant proteins for a high-level and rapid production of foreign proteins in bacteria were developed [16,17]. Size-exclusion chromatography (SEC) for the removal of denaturants and the separation of folding intermediates has been extensively used on refolding proteins that is difficult to refold by conventional methods since 1990s [18,19].

In this study, we seek to clone and express the truncated EGFR extracellular domain (EGFR-tED, 1–501 residues) in *E. coli* as inclusion bodies and screen a novel scFv against EGFR from a human synthetic antibody phage display library. This provides the potential for the development of human antibody-targeting tumor therapy.

Table 1
List of buffers.

Buffer	Buffer preparation
Lysis buffer	50 mM Tris–HCl, 5 mM EDTA, 1%TritonX-100, 1 mM PMSF, 1 mM β-ME, pH 8.0
Washing buffer A	50 mM Tris–HCl, 10 mM EDTA, 1 mg/mL DOA, 1 mM β-ME, pH 8.0
Washing buffer B	50 mM Tris–HCl, 10 mM EDTA, 5 mg/mL DOA, pH 8.0
Washing buffer C	50 mM Tris–HCl, 5 mM EDTA, 4 M urea, 500 mM NaCl, pH8.0
Denaturing buffer	50 mM Tris–HCl, 6 M Gdm–HCl, 100 mM β-ME, pH 8.0
Purification buffer A	20 mM MES, 8 M urea, 500 mM NaCl, pH 5.6
Purification buffer B	20 mM MES, 8 M urea, 500 mM NaCl, 800Mm imidazole, pH 5.6
Refolding buffer A	50 mM Tris, 1 mM EDTA, 3 mM GSH, 1 mM GSSG, 1 M urea, 150 mM NaCl, pH 8.9
Refolding buffer B	50 mM Tris, 1 mM EDTA, 3 mM GSH, 1 mM GSSG, 8 M urea, 150 mM NaCl, pH 8.9
Storing buffer	50 mM Tris, 1 mM EDTA, 150 mM NaCl, pH 8.9

2. Materials and methods

2.1. Cells and reagents

A431 cells, an EGFR-overexpressed epidermoid carcinoma cell line, were purchased from Shanghai Institute of Cell Biology in the Chinese Academy of Sciences, and maintained in DMEM containing 10% newborn calf serum (Gibco). Cell culture was maintained at 37 °C in a 5% CO_2 humidified incubator.

Griffin.1 library is a large naive human scFv phage library, derived by recloning synthetic heavy and light chain variable genes (VH and VL) from human synthetic Fab lox library vectors [20] into the phagemid vector pHEN2 [21]. ScFvs can be expressed as soluble fragments containing c-Myc tag and His-tag, or can be displayed on the surface of bacteriophage when expressed in *E. coli* TG1. ScFv AK404, an anti-VEGFR2 antibody fragment screened from Griffin.1 library by colleagues, was produced at our laboratory. ScFv 11F8 derived from IMC-11F8, a high affinity antibody fragment directed against EGFR, was produced at our laboratory as previously reported by ImClone Systems [9,10].

2.2. Cloning and expression of EGFR-tED

Total mRNA was isolated from A431 cells with a FastTrack kit (Invitrogen). The gene of EGFR-tED was amplified with SuperScript One-step RT-PCR (Invitrogen) and cloned into pMD18-T (TAKARA) vector. The positive clones were selected and confirmed by DNA sequencing. The recombinant plasmid was digested with NdeI and BamHI and inserted into the pET28a(+) (Novagen) expression vector, and then, introduced into *E. coli* BL21 (DE3), identified by double digestion and DNA sequencing. The transformed *E. coli* BL21 (DE3) was cultured in LB medium with 50 μg/mL kanamycin at 37 °C with further induction by 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) for 4 h, when the optical density 600 (OD600) reached 1.0. The cells were preserved at –20 °C after centrifugation at 6000 × *g* for 10 min at 4 °C.

2.3. Pre-treatment of EGFR-tED inclusion bodies

E. coli cell paste (1 g wet weight) was thawed in 20 mL of lysis buffer (Table 1) containing 0.1 mg/mL lysozyme, and disrupted by sonication. Crude inclusion bodies of EGFR-tED were recovered with 20-fold volume of washing buffer A (Table 1) with agitation overnight at 25 °C, and then, washed with 20-fold volume of washing buffer B and C (Table 1) in turn. The final inclusion body pellet was solubilized in denaturing buffer (5 mL buffer per 1 g wet weight inclusion bodies) (Table 1) and incubated for 1 h in a shaker at 37 °C. The supernatant was filtrated with hydrophilic polyethersulfone membrane (0.22 μm, Millipore) after centrifugation at 1,0000 × *g* for 30 min. The harvested denatured EGFR-tED protein supernatant was stored at 4 °C for further treatment.

2.4. Purification of denatured EGFR-tED with gel filtration and IMAC

A sample of 1 mL of denatured EGFR-tED protein was directly applied to a 1 × 100 cm Sephacryl 200HR (a 34 μm average diameter highly cross-linked agarose base matrix with an exclusion limit about 250 KD for globular proteins, GE Healthcare) column equilibrated with purification buffer A (Table 1). The peak contained EGFR-tED protein was collected for the subsequent nickel-based affinity purification with Biologic Duo flow FPLC system (Bio-Rad). The collected sample of 50 mL was applied to a 5 mL HisTrap chelating column (GE Healthcare) equilibrated with purification buffer A (Table 1). A stepwise elution chromatography process was proceeded with mixed buffers of purification buffer A and B (Table 1), containing 100 mM, 200 mM and 500 mM imidazole. The sample eluted by buffer containing 200 mM imidazole was collected, analyzed by SDS–PAGE and stored in 4 °C for refolding experiments.

2.5. Refolding of denatured EGFR-tED by urea gradient size-exclusion chromatography

A Sephacryl S-200 gel column mentioned above was equilibrated with refolding buffer A (Table 1), followed by a gradual increase of refolding buffer B (Table 1) up to 100%. Consequently, a urea gradient was formed in a total volume of 25 mL (Fig. 1), with a urea concentration of 8 M at the top of the column. Afterwards, 700 μL purified EGFR-tED sample was loaded into the column and eluted with refolding buffer B. Equilibration, loading and elution were carried out at 6 °C and the collected protein solution was placed at 25 °C for 20 h [18]. After dialysis against storing buffer (Table 1), the renatured EGFR-tED protein was stored at –70 °C.

2.6. Identification of EGFR-tED by western blotting

The purified EGFR-tED was separated by 12% SDS–PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore). The membrane was blocked in blocking buffer [5% skim milk in phosphate buffered saline solution–0.05% Tween20 (PBS–Tween)] (4 °C, overnight) and incubated with mouse anti-His-tag monoclonal antibody (Millipore) (1 h, 37 °C), followed by peroxidase-conjugated goat anti-mouse IgG (Enogene) (1 h, 37 °C).

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