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Periplasmic expression optimization of VEGFR2 D3 adopting response surface methodology: Antiangiogenic activity study

Wanlu Cao [1], Haixin Li [1], Juan Zhang *, Daojuan Li, Desmond Omane Acheampong, Zhiguo Chen, Min Wang *

State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing 210009, PR China
School of Life Science & Technology, China Pharmaceutical University, Nanjing 210009, PR China

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ABSTRACT

Vascular endothelial growth factor (VEGF) is one of the most significant mediators of angiogenesis, which interacts with a specific membrane receptor: VEGF receptor 2 (VEGFR2). Studies elsewhere have shown that, a VEGF-blocker can regulate several vital processes of tumor promotion. However, there is no literature evidence of investigation on antiangiogenic ability of single domain 3 of VEGFR-2 (VEGFR2 D3), as the key domain in signal transduction of VEGF. In this article, we aimed at developing an efficient method for producing soluble form of this receptor as therapeutic applications. The optimization of the production of soluble VEGFR2 D3 in *Escherichia coli* was firstly done by testing the periplasmic expression in different expression systems using three osmotic shock methods. To enhance the yield, vital factors were selected from nine factors by Plackett–Burman design and the level of each viral factor was optimized via a response surface methodology based central composite design. After purification and identification of the protein, the bioactivity assays: quantitative ELISA, VEGF-induced proliferation and *in vivo* chick chorioallantoic membrane assay were employed in our study. The outcome showed that, *E. coli* Rosetta-gami (DE3)/pET22b-VEGFR2 D3 was the most effective expression system. Furthermore, the inducing time, peptone and glycerol concentration affected the periplasmic expression of VEGFR2 D3 significantly. The corresponding level was also optimized. The bioactivity assay studies showed VEGFR2 D3 could suppress both VEGF stimulated cell proliferation *in vitro* and neovascularization *in vivo*. We have therefore provided a novel antiangiogenic drug candidate relating to VEGF-VEGFR2 pathway.

Introduction

Angiogenesis is a precise and complex pathophysiological process in which pre-existing endothelial cells must break through the basement membrane, migrate and proliferate, corresponding to sundry angiogenic factors. Every step of the process is the result of a highly controlled balance of positive or negative modulators, which are secreted by different cell types or cell membrane adhesion molecules [1].

Vascular endothelial growth factor (also known as VEGF-A)[2] is one of the most significant mediators of angiogenesis. It interacts with a specific membrane receptor: vascular endothelial growth factor receptor 2 (VEGFR2) which expresses in most adult vascular endothelial cells and circulating endothelial progenitor cells. The VEGF-VEGFR2 interaction can activate several intracellular pathways, containing endothelial cell proliferation, migration, differentiation, tube formation, vascular permeability increase and the promotion of integrity [2]. The human VEGFR-2 gene encodes 1356 amino acids, including an extracellular region with seven immunoglobulin-like (7-Ig) domains. Among the seven domains, domains 1–3, domain 3 and domains 5 have been proven to be vital in the VEGF-related signal transduction [3].

VEGF blockers have been researched over decades, which promoted antiangiogenic activity by increasing their affinity with VEGF [4–6]. The blocker can regulate several important processes of tumor promotion and progression, which have been implicated in metastatic colorectal cancer, non-small cell lung cancer, glioblastoma multiforme and vascular eye diseases, notably the wet or neovascular form of age-related macular degeneration (AMD) [7].

However, there is no literature investigation on the pharmacological inhibition ability of single domain 3 of VEGFR-2 (VEGFR2 D3), which is the key domain in the signal transduction of VEGF.

* Corresponding authors. Address: State Key Laboratory of Natural Medicines, China Pharmaceutical University, 154#, Tong Jia Xiang 24, Nanjing 210009, PR China. Fax: +86 25 83271395 (J. Zhang).
E-mail addresses: juancpu@126.com (J. Zhang), minwang@cpu.edu.cn (M. Wang).

[1] These authors contributed equally to this work.

[2] *Abbrevation used:* VEGF-A, Vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor; AMD, age-related macular degeneration; EGF, Epidermal growth factor; FGF, fibroblast growth factors; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; BSA, Bovine serum album; PCR, polymerase chain reaction; TBS, Tris-buffered saline.

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Considering the therapeutic potential of VEGFR2 D3, we sought to develop an efficient and reliable method for producing recombinant VEGFR2 D3 and investigated its potential as a novel antiangiogenic drug candidate relating to VEGF-VEGFR2 pathway.

Material and method

Bacterial strains and plasmids

A strain of *Escherichia coli* (*E. coli*) BL21 (DE3)/pET32a-VEGFR2 D3 preserved in our laboratory was used as the host strain of the VEGFR2 D3 gene. The VEGFR2 D3 gene was cloned into the expression vector pET22b (+) (Novagen).

Reagents

Chemicals, yeast extracts and tryptone were purchased from Merck (Germany); ampicillin was from Sigma (Germany); and isopropyl-β-D-thiogalactopyranoside (IPTG) was from Cinnagen (Iran). Epidermal growth factor (EGF), fibroblast growth factors (FGF), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) were purchased from SBI (Sino Biological Inc., China). Bovine serum album (BSA) was purchased from Sangon Biotech Inc. (China). Others were marked in passages directly.

Subcloning of the VEGFR2 D3 gene and construction of pET22b-VEGFR2 D3

The VEGFR2 D3 gene (Genebank accession No. AF035121) without signal peptide was inserted into a vector of pET22b (+) (Novagen). First, the VEGFR2 D3 gene was obtained by polymerase chain reaction (PCR) conducted in a 50 μl reaction mixture, each containing 2 μl culture of *E. coli* BL21(DE3)/pET32a-VEGFR2 D3 as template, 1 unites of Taq DNA polymerase (Thermo), 5 μl 10× buffer, 2.5 mM of each dNTP, and 20 pmol of the following primers: upstream primer: 5′-CCG GAA TTC (*Eco*RI) TGT GCT GTT CTT CTT GG-3′, downstream primer: 5′-CCG CTC GAG (*Xho*I) GGT AGA ATT TTT CTT CGT CAT-3′. Then, the recombinant vector of pET22b-VEGFR2 D3 was transferred into *E. coli* DH5α. To confirm the structure of new recombinant plasmid, restriction endonuclease digestion and DNA sequencing were carried out.

Comparison of four VEGFR2 D3 expression systems

The optimization of the production of soluble VEGFR2 D3 in *E. coli* was done by testing different expression systems. Both pET22b-VEGFR2 D3 and pET32a-VEGFR2 D3 plasmids were transformed into *E. coli* Rosetta-gami (DE3) and *E. coli* BL21 (DE3) separately, adopting the Ca^{2+} method [8]. The pET system manual (Novagen) was used as reference to express the recombinant protein in the following four expression systems: *E. coli* Rosetta-gami (DE3)/pET22b-VEGFR2 D3, *E. coli* Rosetta-gami (DE3)/pET32a-VEGFR2 D3, *E. coli* BL21 (DE3)/pET22b-VEGFR2 D3 and *E. coli* BL21 (DE3)/pET32a-VEGFR2 D3.

To evaluate whether the target protein was expressed successfully, a signal clone was inoculated in Luria–Bertani (LB) broth with corresponding antibiotics and a shock at 37 °C for 12–16 h (OD_{280} > 1.0) for each of the four expression systems separately. A starter culture was then transferred into 100 ml Erlenmeyer flasks with 20 ml LB broth containing antibiotics. The culture was induced with 1 mM IPTG at 37 °C to express the target protein. The negative control test was carried out with the recombinant strain without adding inducer. Finally, 40 μl of each culture was harvested and tested for protein expression by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The gel was stained with 0.1% (W/V) Commassie's Brilliant Blue R-250 and analyzed with a gel image system (Bio-Rad).

To confirm whether it contains soluble expression, two hundred milliliter (200 ml) of the culture was centrifuged at 8000 rpm for 15 min at 4 °C. Then, the collected cell pellet was suspended in 10 ml of cell lysis buffer (50 mM Tris–HCl, 0.5 M NaCl, 1% Triton X-100 and at pH 8.0) with appropriate lysozyme. The mixture was then centrifuged at 14,000 rpm for 30 min at 4 °C to get the supernatant as soluble expression protein after sonication (10 min). Finally, SDS–PAGE was adopted to test the soluble expression.

Localization of VEGFR2 D3 in different fractions

The analysis of the expression location of target protein was done by preparing four cell fractions according to the following four steps. Firstly, 200 ml culture was centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was then collected as secretary expression. Secondly, the residue was suspended in 10 ml of hypertonic solution (50 mM Tris–HCl, 18% sucrose, 0.1 mM EDTA, and pH 8.0) for 10 min, and centrifuged at 10,000 rpm for 30 min to collect supernatant A. The residue was re-suspended in 10 ml hypotonic solution (5 mM $MgSO_4$) for 10 min, and centrifuged at 10,000 rpm for 30 min to collect supernatant B. The supernatants A and B were mixed, and NaCl added to reach the final concentration of 1.5 M, as the periplasmic fraction. Thirdly, the cell pellet was re-suspended with 20 ml of cell lysis buffer (50 mM Tris–HCl, 0.5 M NaCl, 1% Triton X-100 and pH 8.0) and appropriate lysozyme added. After 10 min sonication, the mixture was then centrifuged at 4 °C for 30 min at 14,000 rpm to collect the supernatant as soluble cytoplasmic fraction. Finally, the residue was dissolved in 10 ml of 8 M urea buffer for 6 h as an inclusion body fraction. All these different fractions were analyzed by SDS–PAGE, followed by Coomassie brilliant blue R-250 staining.

Osmotic shock method

In order to get periplasmic soluble protein, three osmotic shock methods were compared. Since several researchers investigated the optimization of osmotic shock method [9,10], our research focused on the optimized method's practical usage. The details of the three methods are listed in Fig. 1. Method A is an optimized method reported by Ramakrishnan [9]. Method B is a simplified method published by Shouchun Cao [10]. Method C is another method optimized by our laboratory. The main difference between the three methods lies in hypertonic solution and hypotonic solution. The evaluation standards were recorded in two parts: (1) the target protein's yield and (2) the entire collected periplasmic protein's yield. The entire protein yield was tested by Bradford protein assay and the target protein's percentage was obtained by the Automatic Analysis System of Electrophoresis Gel Imaging (Bio-Rad). The target protein's yield equaled the target protein's percentage multiply by the entire collected periplasmic protein's yield. All experiments were repeated three times to gain the average.

Plackett–Burman design (PBD)

The optimization of the periplasmic expression of VEGFR2 D3 in *E. coli* Rosetta-gami (DE3) was done by employing PBD [11,12]. Nine variables were carefully selected and evaluated by PBD with twelve experiments. These variables were: induction time, induction temperature, rotational speed after induction, IPTG (inducer) concentration, glycerol concentration, yeast and peptone concentration, NaCl concentration and ammonium sulfate. The experiments were designed and analyzed with the software package "Minitab 15" (Minitab Inc). The PB experiments contained a total