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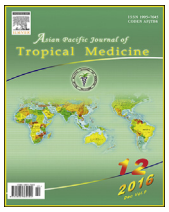

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Recombinant expression and purification of functional vascular endothelial growth factor-121 in the baculovirus expression system

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ABSTRACT

Objective: To express human vascular endothelial growth factor121 (VEGF121) in insect cells.

Methods: A gene construct containing VEGF was cloned in the pFastBac-HTA vector, followed by transformation in DH10BAC. The recombinant bacmid was then extracted, and transfected into Sf9 insect cells. The transfected cells were harvested, and then VEGF expression was confirmed by western blotting using specific antibodies. The tube formation assay was used for functional assessment of VEGF.

Results: Our results showed that VEGF could be successfully expressed in the baculovirus system. Purified VEGF was able to stimulate *in vitro* tube formation of human endothelial cells.

Conclusions: Results from this study demonstrated that the recombinantly-produced VEGF can be considered as a promising candidate for therapeutic purposes.

1. Introduction

Angiogenesis refers to the formation and development of new blood vessels from pre-existing blood vessels [1]. Angiogenesis is a rare phenomenon in healthy adult, which only occur temporarily under specific physiological conditions such as wound healing, inflammation and women's sexual cycle [2,3]. A disruption in the balance between angiogenic and anti-angiogenic factors leads to pathological angiogenesis [4–6]. There is strong evidence that tumor cells need new blood vessels to grow, invasion and spread [7,8]; in the absence of blood vessels, tumor cells can not exceed more than 1–2 mm in size [9]. Vascular endothelial growth factor (VEGF) is the most important angiogenic factor [4,10–12], which includes 5 members in mammals such as VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGE (Placental growth factor). VEGF-A, the most important member of this family [13], interacts with two receptors, VEGFR-2 and VEGFR-1, resulting in endothelial cell proliferation and angiogenesis [14,15]. VEGF-A consists of 5 main isoforms with different amino acid residues, including VEGF121, VEGF145, VEGF165, VEGF189 and VEGF206. Importantly, VEGF121 and VEGF165 are the most abundant isoforms with 121 and 165 amino acid residues, respectively [16–18]. These isoforms are found in the majority of cells expressing the VEGF gene. The presence of exon 7 in the VEGF165 gene, but not in the VEGF121 gene, enables VEGF165 to bind to heparin, heparan sulfate and neuropilin-1(NP-1) receptor, which play a role in the development of embryo [16]. The low molecular mass of VEGF121 makes it soluble and freely diffusible [19]. A variety of studies demonstrated that VEGF121 displays full biological activities of larger isoforms [19], emphasizing the role of VEGF121 as a potential target for drug development. Difficulties in the production of recombinant proteins, such as VEGF, in *Escherichia coli* (*E. coli*) lead to the development of novel expression systems such as the baculovirus expression system [20]. Insect cells are

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demonstrated to be an excellent alternative for the production of animal proteins [21]. By definition, insect cell expression systems that use insect viruses are considered as a baculovirus. Simply stated, baculoviruses are a group of viruses that infect vertebrates and insects [22]. One of the important advantages of these systems over bacterial expression systems is to mediate post-translational modifications such as phosphorylation, glycosylation and acylation. The formation and reduction of disulfide bonds (proteolytic characteristics) result in the production of recombinant proteins, which is structurally similar to natural proteins. Another important benefit of this system is to allow low-cost production of recombinant proteins [23]. In this regard, the aim of this study was to express VEGF in the baculovirus system.

2. Material and methods

2.1. VEGF121 DNA construct preparation

The human VEGF121 gene was cloned in pET26b in our previous study [24]. In the present study, we attempted to clone hVEGF into the pFastBac HTA transfer plasmid (Bac-to-Bac expression system). For this purpose, the hVEGF gene was amplified with specific primers anchored with *BamH*I and *Xho*I restriction enzyme sites (Table 1). Amplified hVEGF was cloned in the pFastBac HTA vector. Subsequently, the construct was transformed into *E. coli* TG1 competent cells, and confirmed. The fidelity of cloned sequences was verified by DNA sequencing; the resulting construct was named pFast-hVEGF (Figure 1A).

Table 1
Primers sequence.

Primer names	Primer sequences
hVGF-BacF (*Bam*HI)	5′-acgGGATCCGGCACCCATGGCAGAAG-3′
hVGF-BacR (Stop-*Xho*I)	5′-acgCTCGAGTTACCGCCTCGGCTTG-3′
M13 F	5′-GTTTTCCCAGTCACGAC-3′
M13 R	5′-CAGGAAACAGCTATGAC-3′

2.2. Transposition of the VEGF121 gene to baculovirus bacmid

The pFast-hVEGF construct was transformed into DH10Bac bacteria to establish recombinant baculovirus bacmid (Bac-to-Bac baculovirus expression system). In the Bac-to-Bac baculovirus expression system, transposition generally occurs between two areas of mini-Tn7 pFastBac and mini-att Tn7 baculovirus Bacmid. Replacement of mini-Tn7 at the junction of Bacmid results in LacZ disruption. Therefore, the recombinant Bacmid was screened after 48 h using blue–white colony screening on LB agar containing 50 μg/μL kanamycin, 25 μg/μL tetracycline and 40 μg/μL gentamicin, and incubated at 37 °C for 18 h. Fidelity of the VEGF gene transferred into Bacmid was confirmed by PCR using M13 primers (Table 1). The recombinant bacmid was extracted from DH10Bac according to the standard method [25], and used for transfection of Sf9 insect cells.

2.3. Sf9 culture and transfection

Sf9 cells (obtained from National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran) were cultured in Grace's insect medium (Invitrogen, gibco) supplemented with 10% FBS, 100 U/mL of penicillin, 100 mg/mL of streptomycin, and incubated at 27 °C in a humidified incubator. The recombinant bacmid construct was transfected to exponentially-growing Sf9 cells using a cellfectin (Invitrogen) transfection reagent according to the manufacturer's instructions.

2.4. Recombinant baculovirus preparation

Cytopathic effects (CPEs) were detected 4 days after transfection. The supernatant of Sf9 cells was collected (P1 or First Generation of virus), and used to infect new Sf9 cells. The P1 virus was used for Sf9 transfection to establish P2 and P3. To confirm VEGF expression, cells transfected by P1, P2 and P3 were harvested after four days of infection. Then, expression of recombinant VEGF121 was evaluated by SDS-PAGE and western blotting on cell lysates. Avastin (Commercial Anti-VEGF, at a dilution of 1:4000) and Anti-Human IgG HRP-conjugated (at a dilution of 1:4000) were used as primary and secondary antibodies in western blot analysis, respectively.

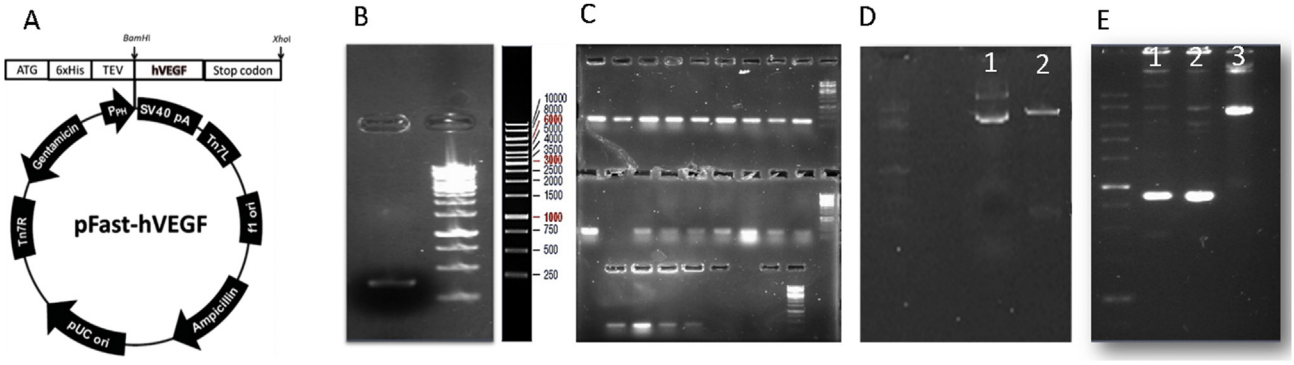

Figure 1. A) Schematic structure of the VEGF gene in the plasmid pFastBac HTa. B) Amplified hVEGF121 (400 bp). C) Colony-PCR results (after cloning of $hVEGF_{121}$ in pFastBac HTA plasmid). D) Double digestion of pFast-hVEGF resulting plasmid with *Bam*HI and *Xho*I. Lane 1, undigested and lane 2, double digested. E) DNA electrophoresis of isolated recombinant bacmid (lanes 1, 2) and non-recombinant bacmid (lane 3) with lambda phage marker.

2.5. Purification of the recombinant protein

For VEGF purification, a density of approximately 6×10^7 Sf9 transfected cells were harvested, and then suspended in 5 mL PBS. The cells were disrupted by three freeze/thaw cycles, and subsequently suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole), followed by 15 ultrasonication treatments of 15 s each. The mixture was centrifuged at 6000 × g for 30 min at 4 °C to remove insoluble particles. Supernatants were filtered using 0.45 μm syringe filters, and then loaded on the nickel affinity column (QIAGEN, Germany). The column was washed using washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole) to elute unbound proteins. The recombinant His-tagged VEGF was eluted using elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole). The purification process was confirmed by 15% SDS-PAGE and western blot analysis. Expression levels were detected by the Bradford assay.

2.6. Tube assay

For tube formation assay, we used primary HUVECs (Human Umbilical Vein Endothelial Cells) between passages 2 and 6, as isolated in our previous study [26]. A solution of Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane (Gibco, Invitrogen) was thawed at 4 °C, coated at a dose of 50 μL on a 96-well plate, and incubated at 37 °C in a humanified incubator for 30 min in order to solidify the Geltrex. 100 μL of EBM-2 medium containing 5000 HUVEC cells with and without of recombinant expressed VEGF was added to each well. After 2, 4 and 6 h, cell conditions and tube formation were monitored under an invert microscope (INV100-FL, BEL-Italy). In addition, it is important to note that the assay was carried out in triplicate.

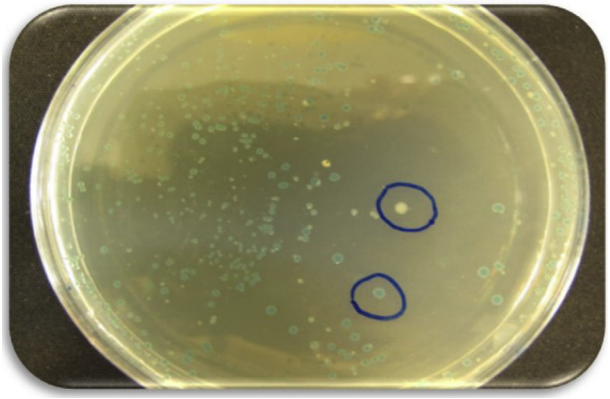

Figure 2. Blue–white colony screening for transformation of pFastBac-VEGF in DH10Bac.

3. Results

3.1. hVEGF121 amplification and recombinant baculovirus DNA preparation

As shown in Figure 1B, the amplified human VEGF121 (400 bp) was detected in gel electrophoresis. hVEGF121 and pFastBac were digested with *Bam*HI and *Xho*I, ligated and transformed into *E. coli* TG1 competent cells. Approximately 24 colonies were screened by colony-pcr, 21 colonies of which contained recombinant hVEGF121 (Figure 1C). In addition, *Bam*HI and *Xho*I double digestion (Figure 1D) and sequencing confirmed the fidelity of recombinant hVEGF121. Finally, recombinant pFast-hVEGF121 was transformed into DH10Bac, and recombinant baculoviral DNA resulted from the Bac-to-Bac baculovirus expression system. Results from PCR, carried out on baculovirus plasmid, showed that recombinant baculovirus yielded a band of 2740 bp (Figures 1E and 2E).

3.2. Transfection of Sf9 cells

Transfection of Sf9 cells was confirmed by the recombinant baculovirus (Figure 2). After 72-h post transfection, cytopathic effects (CPEs) were found in transfected Sf9 cells as compared to non-transfected Sf9 cells (control cells), indicating the successful transfection (Figure 3A). The cells transfected with recombinant baculovirus showed CPEs of nucleus enlargement and granulation (Figure 3B).

3.3. Expression and purification of recombinant hVEGF

After 3 days of Transfection when CPEs were observed, Sf9 cells were harvested, and expression of hVEGF121 was evaluated by using 15% SDS-PAGE and western blot analysis. As

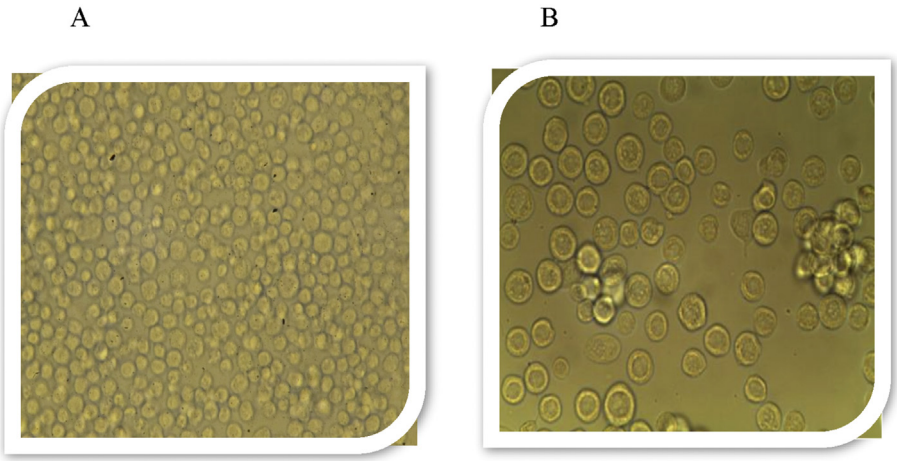

Figure 3. Transfection of Sf9 cells by recombinant bacmid DNA. A; non-transfected Sf9. B; Sf9 cells were transfected by recombinant baculovirus, and cytopathic effects (CPEs) were detected after 72 h of transfection.

shown in Figure 4A, hVEGF121 was expressed in transfected cells; two protein bands (15–40 kDa) indicate different glycosylated forms of the protein. For hVEGF121 purification, 6×10^7 of Sf9 cells were disrupted by sonication, and cell debris was removed by centrifugation. The supernatant was loaded on the Ni-NTA column, and recombinant protein was eluted by imidazole 250 mM. The yield of purified recombinant VEGF121 per liter of Sf9 cells was about 500 μg.

3.4. Tube formation assay

The findings from this study indicated that recombinant VEGF121 has the ability to increase tube formation in HUVECs. As shown in Figure 5, VEGF121-treated HUVECs were able to form tube-like structures. By contrast, there were no tube like structures in control cells (HUVECs with no VEGF). The cells were stained with calcein AM (Trevigen) according to the manufacturer's protocols.

4. Discussion

Nowadays, there is a worldwide demand for recombinant protein production, as one of the main interest of pharmaceutical industries [27]. The most important decisions in the selection of recombinant protein production and purification are related to the host that affects product quality. To choose an appropriate host, some important criteria must be considered, including the amount of product required, toxicity, ease of purification and biological activity of the product [28]. There had been several expression systems, each with its own advantages and disadvantages. Today, the *E. coli* expression system is the most commonly used expression system for recombinant protein production. The best-known benefits for this system are the ability to grow fast, simple and cheap requirements, high-density environments, well-known genetics and molecular biology, and the capability to express high levels of recombinant proteins [29,30]. However, There are some critical disadvantages for this

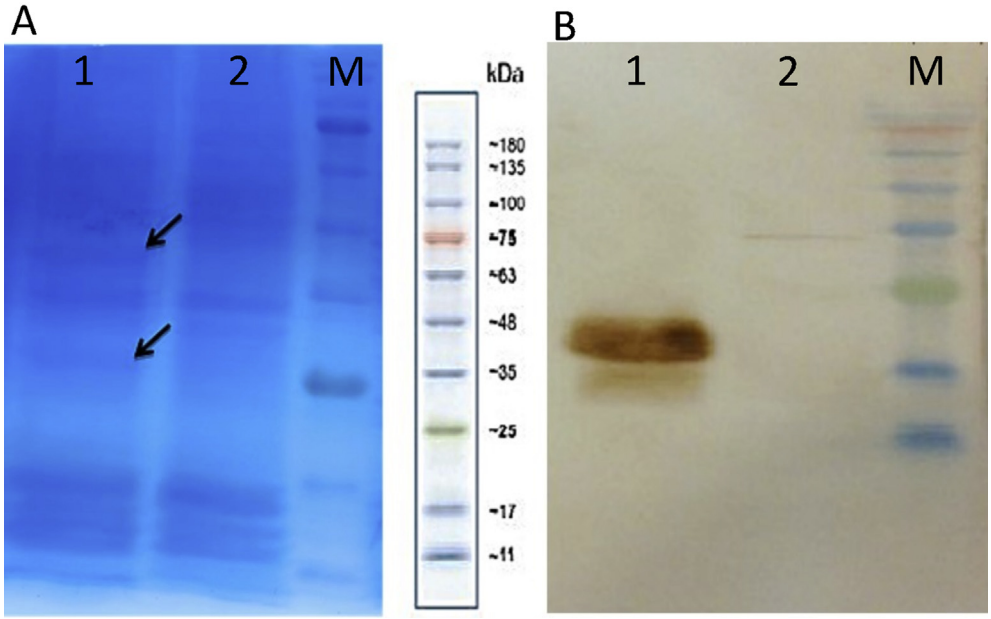

Figure 4. A) SDS-PAGE results, 1; transfected cells, 2; non-transfected cells. As it can be seen, there is two bands in transfected Sf9 cells (lane 1) showing different glycosylation patterns. B) Western blot analysis with bevacizumab antibodies. lane 1: the purified hVEGF121, lane 2: the negative control. M; protein marker.

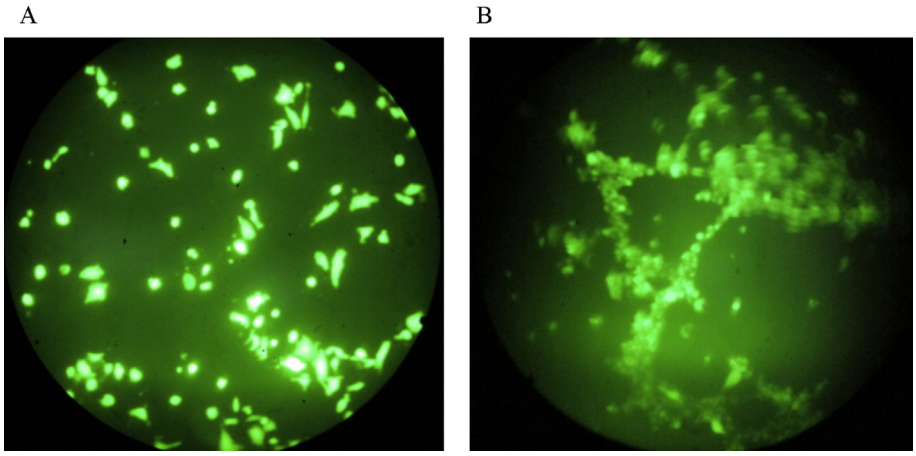

Figure 5. Tube assay result. A) The lack of VEGF. B) The presence of VEGF. Tube-like structures were formed in the presence of VEGF as a simulator.

system, including the inability to perform post-translational modifications (such as glycosylation), improper recombinant protein folding and secretory mechanisms, as well as inefficient or low efficient disulfide bond formation. Another useful expression system is a mammalian expression system. Although protein produced in mammalian expression systems lacks the limitations of bacterial expression system, the main disadvantage of mammalian systems is their need for expensive cell culture [31]. However, baculovirus is one of the common systems for recombinant production of proteins. Here, for the first time, we successfully expressed hVEGF121 in the baculovirus system. In addition, the ability of VEGF to stimulate endothelial cells was evaluated by the Tube formation assay.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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