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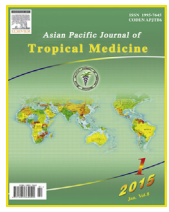

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Recombinant expression and purification of functional vascular endothelial growth factor-121 in the baculovirus expression system

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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 by colony-PCR with POLH-F and PFSBC-R primers (Table 1). 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 (BamHI)	5′-acgGGATCCGGCACCCATGGCAGAAG-3′
hVGF-BacR (Stop-XhoI)	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 in an atmosphere of 5% CO_2. 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 HRP-conjugated Anti-Human IgG (at a dilution of 1:4000) were used as primary and secondary antibodies in western blot analysis, respectively.

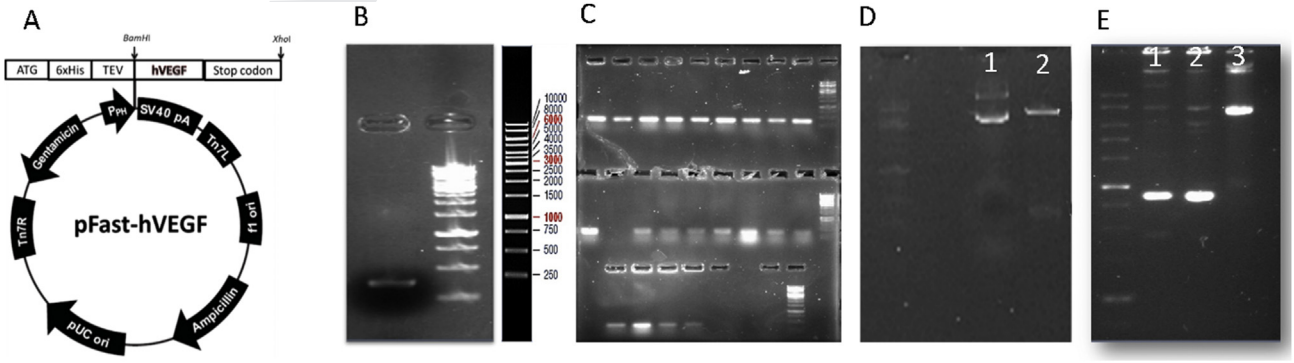

Figure 1. A) Schematic structure of the VEGF gene in the plasmid pFastBac HTa. B) Amplified hVEGF121 (400 bp). C) Colony-PCR results (after cloning of $hVEGF_{121}$ in pFastBac HTA plasmid). D) Double digestion of pFast-hVEGF resulting plasmid with *Bam*HI and *Xho*I. Lane 1, undigested and lane 2, double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