

Research Article

Expression and Purification of Functional Human Vascular Endothelial Growth Factor-A₁₂₁; the Most Important Angiogenesis Factor

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Abstract

Purpose: Angiogenesis or formation of new blood vessels is an essential process for tumor growth, invasion and metastasis. Vascular Endothelial Growth Factor (VEGF) and its receptors play an important role in angiogenesis-dependent tumors. VEGF-A is the most important factor in angiogenesis process. Human VEGF-A gene consists of eight exons that undergoes alternative exon splicing and produce five different proteins consisting of 121, 145, 165, 189 and 206 amino acids (named VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆).

Methods: In this study, VEGF₁₂₁ gene synthesized and cloned into the pET-26b plasmid. The recombinant plasmid was transferred into appropriate expression strain of BL-21. Expression of VEGF₁₂₁ induced by IPTG (Isopropyl β-D-1-thiogalactopyranoside) and confirmed by SDS-PAGE and Western-Blotting. Recombinant VEGF₁₂₁ was purified by nickel affinity chromatography. HUVECs (Human Umbilical Vein Endothelia Cells) cells were isolated from umbilical vein and the effect of VEGF₁₂₁ on tube formation of endothelial cells was investigated.

Results: SDS-PAGE and Western-Blotting results verified the purification of VEGF₁₂₁. The final yield of recombinant protein was about 5mg per liter. Endothelial cell tube formation assay results showed that VEGF₁₂₁ leads to tube formation of endothelial cell on matrix and induces angiogenesis *in vitro*.

Conclusion: Recombinant VEGF₁₂₁ is important factor in tube formation of endothelial cell, so it could be used in different cancer researches and angiogenesis assay.

Introduction

Angiogenesis is a fundamental process which is tightly regulated and resulted in the formation of new blood vessels from the previous vessel. This process is an essential component in the growth of normal cells and tissues during embryonic development, reproductive cycle and wound healing. Angiogenesis also play a critical role in pathological conditions, such as; age related macular degeneration (AMD), rheumatoid arthritis (RA) and diabetes retinopathies.¹⁻⁵ Vascular Endothelial Growth Factor is the most important regulators of angiogenesis.⁶⁻⁷ VEGF family consist of seven secretory glycoproteins named, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and Placental Growth Factor (PlGF).⁸⁻⁹ The biological action of vascular endothelial growth factors has on its target cells through interactions with receptor tyrosine kinase in the plasma membrane cells conduct.¹⁰ The binding of VEGF and its receptor cause receptor dimmer and autophosphorylation.¹¹ Ultimately these events led to the growth of endothelial cell and angiogenesis. VEGF

ligands induce their effects by binding to three tyrosine kinase receptors -1, -2 and -3 (VEGFR-1, 2, 3).¹²

VEGF-A is the Main factor in the angiogenesis and acts its effect through the activation of VEGFR-1 and VEGFR-2. The human VEGF-A gene consists of eight exons which undergoes alternative exon splicing and results five different proteins consisting of 121, 145, 165, 189 and 206 amino acids (named VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉, VEGF₂₀₆). Exons 1-5 are conserved in all isoforms and alternative splicing occur in exon 6 and 7. VEGF₁₆₅ lacks the exon 6 and is moderately diffusible but VEGF₁₂₁ lacks both exon 6 and 7 and is highly diffusible.¹³ VEGF₁₂₁ and VEGF₁₆₅ are the most physiologically relative isoforms.¹⁴ VEGF₁₂₁ has a disulfide band and form homodimer with molecular weight of 28kDa. The molecular weight of its monomer form is 16 kDa.¹⁵

To evaluate the effect of angiogenic inducers and inhibitors some *in vitro* models have been developed including endothelial tube assay, proliferation assay and scratch assay.¹⁶⁻²² In endothelial tube assay, endothelial

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cells interact with a matrix which helps cells to attached, aligned and form tube-like structure¹⁹ without cell proliferation.²⁰ Because cell tube formation of endothelial cells on basement membrane mimics many steps of the angiogenesis, it has been widely used as a screen for angiogenic and anti-angiogenic factors.²⁰ Since the most important regulators of angiogenesis are vascular endothelial growth factors so to achieve the goal, these factors should be considered.

The goal of this study was cloning, expression and purification of recombinant human VEGF₁₂₁, as well as assessment of its *in vitro* biological activity by endothelial tube formation and proliferation assays.

Materials and Methods

Gene construction of pET26-VEGF₁₂₁

The gene sequence corresponding to the human VEGF₁₂₁ adapted to the genome of *E.coli* BL-21(DE3) and synthesized by Biomatik Co. (Canada). The synthesized gene was amplified by specific primers which anchored with *NdeI* and *XhoI* restriction sites (Table 1). pET-26b plasmid and amplified VEGF₁₂₁ gene were digested with the restriction enzymes and ligated overnight at 16°C. The construct was transformed into *E.coli* BL-21(DE3) competent cells and confirmed by colony-PCR with T7 promoter and T7 terminator primers. The integrity of final construct (pET26-VEGF₁₂₁) was confirmed by double digestion and DNA sequencing.

Table 1. Specific primers of VEGF₁₂₁ with restriction sites (in bold and underlined)

Primer name	Sequence 5' → 3'	Length (bp)
Forward primer	ACG <u>CATATG</u> GGCACCATGGCAGAAG (<i>NdeI</i> site)	26
Reverse primer	ACG <u>CTCGAG</u> CCGCCTCGGCTTG (<i>XhoI</i> site)	22

Recombinant Expression of VEGF₁₂₁

One colony of BL-21(DE3) *E.coli* include confirmed construct inoculated into LB Broth medium. The bacteria in log phase (OD_{600nm} = 0.5-0.9) was induced with 1 mM of IPTG and incubated for 12-14h at 37°C, 180rpm. Expression was confirmed with SDS-PAGE and western blotting. SDS-PAGE was performed with 15% polyacrylamide gel. For western blotting, the protein bands were transferred onto nitrocellulose paper, and was blocked with 4% skim milk for 2 hours, then incubated with first antibody (human anti-VEGF) solution (1/4000) for 1 hour and second antibody (anti-human HRP conjugated) for another 1 hour. The recombinant protein was detected by ECL development (Electro Chemo Luminescence, GE. Healthcare).

Recombinant VEGF₁₂₁ Purification

After overnight induction with IPTG, the bacterial pellet was resuspend in binding buffer (Imidazole 30mM, NaCl 500mM, Tris 50mM and Urea 8M) and incubated for 1 hour in cold room. After that, the suspension was ultrasonicated for 15 second and 15 times (0.5 cycle and amplitude of 1). The remaining cells debris were precipitate by centrifugation at 8,000rpm for 30min. Protein extract from the bacteria were loaded on nickel affinity column (QIAGEN, Germany) according to the method proposed by the company producers. After washing with washing buffer (Imidazole 60mM, NaCl 500mM, Tris 50mM and Urea 4M), the recombinant protein was eluted with Elution buffer (Phosphate Buffered Saline (PBS) with Imidazole 500mM). For refolding, the recombinant protein was dialyzed against PBS. Finally, the purification process was confirmed by 15% SDS-PAGE (Reduced condition) and Western Blotting. The final yield was determined by Bradford assay. The purified VEGF₁₂₁ lyophilized in a freeze dryer system (Christ, 2 alpha - Germany) at -55°C and stored at -20°C for further use.

Isolation of HUVEC cells from umbilical vein

Human umbilical cord collected from Babak hospital, (Tehran, Iran) and transferred to laboratory in transfer buffer (PBS, 0.2% glucose, 0.5M EDTA, 100U/ml penicillin and 100µg/ml streptomycin). The isolation of HUVEC cells from embryonic umbilical vein was done according to Bruno Baudin protocol.²¹ Briefly, umbilical vein was washed with DPBS (D-glucose and PBS) solution. Collagenase type 1 solution (Sigma) was used for isolation of HUVEC cells. After 30 min incubation at 37°C, the cells collected and centrifuged at 2000rpm for 5 minutes. Supernatant was removed and cells pellet resuspended in M200 (Gibco, Invitrogen) culture medium supplemented with 10% heat inactivated FBS (Fetal Bovine Serum) (Gibco, Invitrogen), and low Endothelial Cell Growth Supplement (ECGS, Gibco, Invitrogen), penicillin G 100U/ml (Sigma) and streptomycin 100µg/ml (Sigma), and transferred into 25 cm² culture flask and incubated at 37°C with humidified air containing 5% CO₂. After 3-7 days, cells confluency was checked. The cells detached from culture flask with trypsin-EDTA (Sigma) and used for next experiments.

Cell proliferation assay

About 2×10⁴ HUVEC cells seeded in 24-well plate with 1 ml of M200 medium supplemented with 2% FBS. Next day, the cells were washed with PBS and incubated in serum starvation medium (M200 with 0.1% FBS). After 24 h incubation at 37°C and 5% CO₂, different concentrations of VEGF₁₂₁ (10, 50, 75 and 100 ng/ml) and standard commercial VEGF₁₂₁ from R&D system (10, 50, 75 and 100 ng/ml) were added to the wells and incubated for an additional 24 h. Finally, the cells trypsinized and counted against control well.

Endothelial tube formation assay

Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane (Gibco, Invitrogen) solution was thawed on ice

and 50µl aliquots were transferred into a 96-well plate and incubated at 37°C for 30 minutes to solidify. Hundred microliters of M200 medium containing about 10⁴ HUVEC cells (at least from 2nd passage, but not more than 6 passages) were added to each well on Geltrex™. The cells were treated with 0.1% FBS and VEGF₁₂₁ (75ng/ml). The assay was done in triplicate and plate incubated at 37°C with 5% CO₂. After 4, 6 and 16 hours of incubation, the cells and tube formation conditions observed under invert microscope (INV100-FL, BEL-Italy).

Results

Gene construction and cloning

VEGF₁₂₁ gene was amplified with specific primers which designed for specific sequence with restriction enzymes site at the tails (Figure 1A). The amplified PCR product and pET26b plasmid were digested, ligated and transformed into BL-21(DE3) *E.coli* competent cell. Some colonies were checked by Colony-PCR with T7 promoter and T7 terminator primers (Figure 1B), that all of them had the desired fragment. For further confirmation, *NdeI* and *XhoI* double digestion and sequencing was done (data not shown).

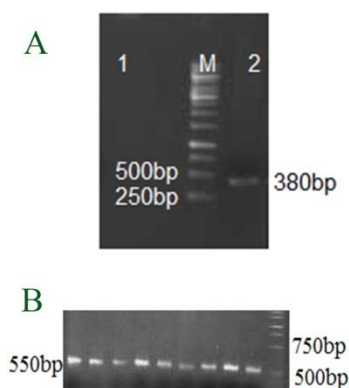


Figure 1. (A) VEGF₁₂₁ gene amplification, (1) negative control, (2) amplified PCR product of VEGF₁₂₁ (380bp) (M) 1 Kb DNA ladder. (B) Colony-PCR for randomly selected transformants. Band at 550bp indicating positive colonies that had recombinant plasmid.

Expression and purification of recombinant VEGF₁₂₁

The recombinant protein was induced with different concentration of IPTG and the best expression was investigated in concentration of 1mM at 37°C for 12-14 hours. Expression was confirmed by SDS-PAGE and Western-Blotting (Figure 2). The recombinant VEGF₁₂₁ was expressed as His-tag fusion protein and purified with nickel affinity chromatography (Figure 2C). The yield of expression was detected by Bradford test and was evaluated about 5mg/liter.

Proliferation assay

As shown in Figure 3, the effect of recombinant VEGF₁₂₁ on HUVECs proliferation was dose dependent. We showed that increasing the concentration of VEGF₁₂₁ cause the increasing trend in HUVECs proliferation. However the increasing trend had a plateau at 75ng/ml of

VEGF₁₂₁. According to the obtained results the concentration of 75ng/ml was used for further investigations. However with standard VEGF₁₂₁, the graph was reached plateau at concentration of 50ng/ml, and in comparison with our recombinant VEGF₁₂₁ its effect is moderately higher than recombinant VEGF₁₂₁ on HUVECs proliferation.

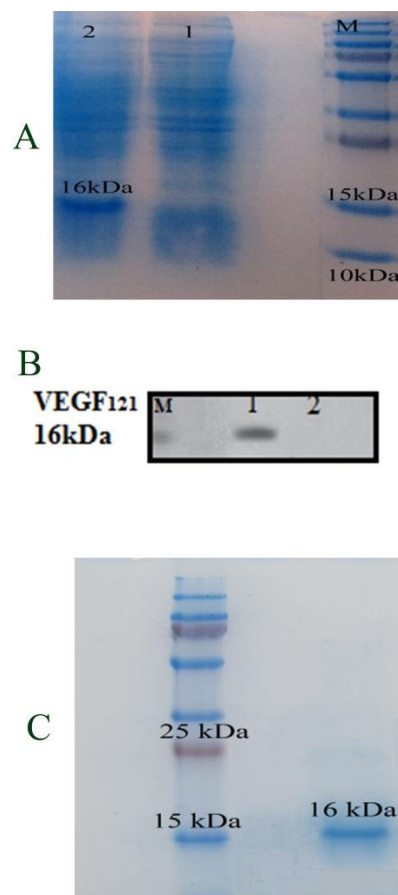


Figure 2. VEGF₁₂₁ expression and purification. (A) Evaluation of VEGF₁₂₁ expression by 15% SDS-PAGE analysis under reduced condition. (1) uninduced and (2) induced with IPTG (M) Protein marker. (B) Western-blot analysis with specific anti-VEGF monoclonal antibody. (1) induced with IPTG and (2) uninduced (C) Purification result of recombinant VEGF₁₂₁ with Ni-NTA method.

VEGF₁₂₁ promote tube formation in HUVECs

The results clearly demonstrated that recombinant VEGF₁₂₁ caused increase of tube formation and branching of tubes in HUVEC cells. As shown in Figure 4, the treated cells with VEGF₁₂₁ forms tube like structures but in control wells (wells supplemented with 0.1% FBS), no tube-like structure were seen (Figure 4).

Discussion

Angiogenesis is a critical multi step process that resulted in the growth and sprouting solid tumor cells. It has been proved that inhibition of angiogenesis, is an effective way for treatment of solid tumors.² Inhibition of angiogenesis is based on the blocking of new blood vessels formation through inhibition of interaction

between vascular growth factors and endothelial cells in tumor tissue.²² The most important vascular growth factor is VEGF, that is specific mitogen of endothelial cells and resulted in metastasis.²³ Interaction of VEGF and its receptors (VEGFR-1 and VEGFR-2) is very important for tumor angiogenesis.²³ So inhibition of VEGF and blocking of its signaling through interaction by its receptors could be promising tool for treatment of cancer.²⁴ Given the importance of VEGF in angiogenesis in pathologic condition, and the widespread use of therapeutic factors in control of tumor progression, the main aim of this work was optimization of VEGF₁₂₁ expression in *E.coli* and applying it as endothelial cell growth factor to studying angiogenesis *in vitro*. According the importance of angiogenesis in the progression of some diseases, including cancer, Rheumatoid arthritis, diabetic retinopathy,¹¹ recombinant production of this factor seems necessary which can induce angiogenesis for studying *in vitro*. Right regulation of angiogenesis is related with right balance between inhibitors and inducers of it. For more than a decade, the role of VEGF in the regulation of angiogenesis has been studied. Some studies have focused on expression of VEGF,²⁵⁻²⁶ whereas in most of them, have been focused on the angiogenesis effect of VEGF₁₆₅. For the first time in Iran, the roll of VEGF₁₂₁

on endothelial cell tube formation and angiogenesis were investigated.

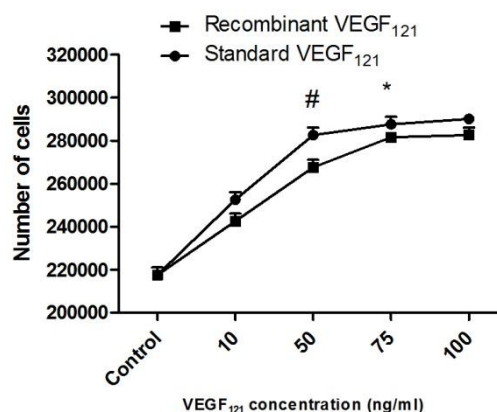


Figure 3. Proliferation effect of recombinant and standard commercial VEGF₁₂₁ on HUVEC cells. Different concentrations of VEGF₁₂₁ (10, 50, 75 and 100 ng/ml) was performed on HUVECs. With increasing the concentration of VEGF₁₂₁, the increasing trend was observed in HUVECs proliferation which reached a plateau at 75ng/ml of VEGF₁₂₁ concentration (indicated by *). This effect is due to saturation of VEGF receptor on HUVECs. But, in case of standard VEGF₁₂₁ was used, the graph in concentration of 50ng/ml was reached to plateau. The assay was performed in triplicate and error bar represent for standard deviation. (mean±SD)

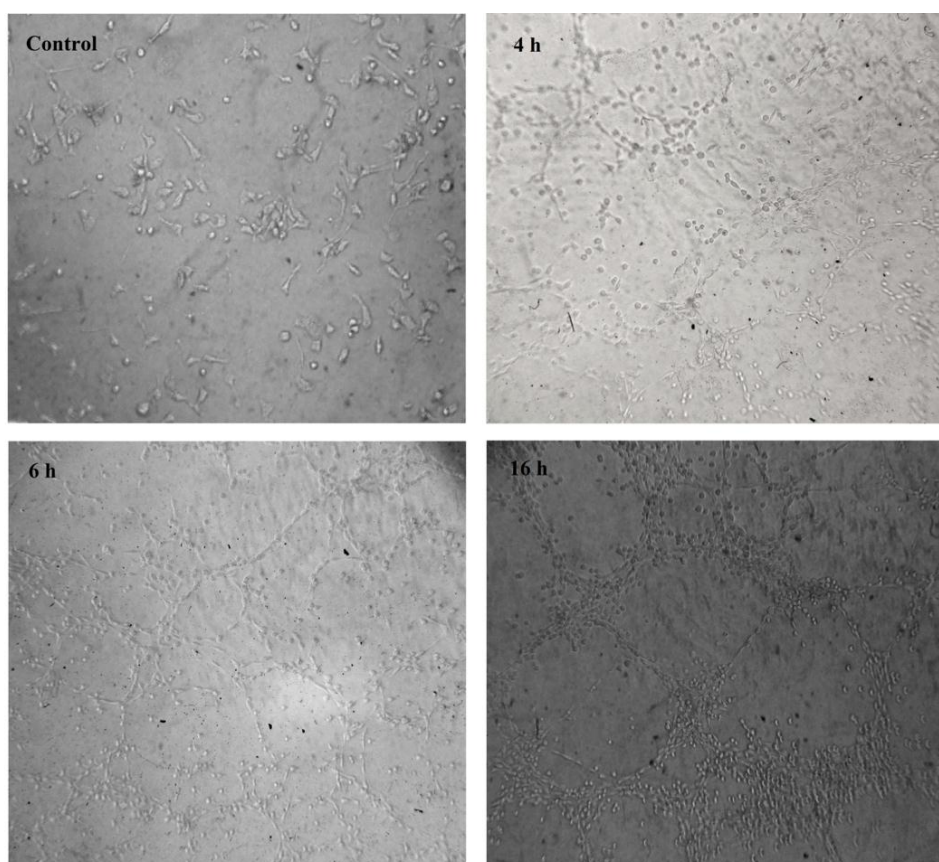


Figure 4. Effect of VEGF₁₂₁ on endothelial tube formation. The assay was followed at 4, 6 and 16 h after treatment with VEGF₁₂₁. The tube formation was visualized through invert microscope (×10). As it can be seen the area of formed tubules increased by increasing time of HUVECs exposure with VEGF₁₂₁ and after 16 h exposure of HUVECs with VEGF₁₂₁ network formation was completed. The assay repeated four times.

Endothelial cell tube formation assay on matrix is as a first screen assay and has many advantages such as; rapidness, quantitative and can be done in high throughput level. Also it include all steps of angiogenic process; adhesion, migration, alignment and tube formation.²⁷ Therefore this assay has many advantages over other assays such as migration, cell attachment, proliferation and invasion, which measure fewer steps in angiogenesis.^{20,28} However, this assay was first described over 20 years ago, but until to date this powerful assay has been widely used for identifying many various angiogenic and anti-angiogenic factors. Optimal result depends on some important conditions, like the number of coated cells. The optimal number of the cells is about 10000-15000 cells/well on 96-well plate. In case of primary cells, to obtain ideal tube formation, the cells must be in early passage (passage 2-6). Moreover the amount of matrix is important too and the best result of tube formation is observed in 50µl/well of 96-well plate.²⁰

Our experiment showed that His-tagged VEGF₁₂₁ was successfully expressed by *E.coli* BL-21(DE3). The total expression of the VEGF₁₂₁ was 5mg per liter of culture. All isoforms of VEGF are glycosylated at Asn74, which is not affecting the VEGF biological activity²⁸. So the BL-21(DE3) is an appropriate host for our aim. According to results, VEGF₁₂₁ resulted HUVECs proliferation in dose dependent manner. With increasing the concentration of VEGF₁₂₁ no significant increasing in HUVECs proliferation was observed at dose higher than 75ng/ml. This phenomena may be indicated the saturation of VEGF receptors. Comparing the result of the recombinant VEGF₁₂₁ with standard commercial VEGF₁₂₁ showed that the effect of standard VEGF₁₂₁ is moderately higher than our purified on HUVECs proliferation. We also showed that VEGF₁₂₁ can induce endothelial tube formation and angiogenesis *in vitro*, in fact VEGF₁₂₁ play an important role in angiogenesis process and endothelial cell tube formation. So this factor suggests the potential for novel therapeutic approaches.

Conclusion

According above mentioned and importance of angiogenesis in research to identify angiogenic and anti-angiogenic factor for treatment of different disease such as cancer, methods that inhibit angiogenesis are a promising rout for the treatment of angiogenesis-dependent disease. The purified VEGF₁₂₁ was biologically active as assessed by proliferation of HUVECs and *in vitro* tube formation. The condition of expression that is explained in this study is expected to be applicable for large-scale production of various VEGF proteins in *E.coli*. Furthermore, VEGF₁₂₁ can be used for research and clinical application in cancer researches.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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