Cloning and Expression of Human Vasohibin1 Gene in E. coli

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Received: 10 May 2016  Accepted: 14 June 2016

Abstract

Background: Angiogenesis is an important process in various physiologic and pathologic states. The most significant stimulator of angiogenesis is vascular endothelial growth factor (VEGF). In contrast, vasohibin1 acts as an angiogenesis inhibitor which specifically inhibits new vessels formation. The aim of the present study was cloning and expression of vasohibin1 gene in E. coli as well as purification of recombinant vasohibin1 protein.

Methods: Total RNA was extracted from human umbilical vein endothelial cells and cDNA was synthesized by RT-PCR. cDNA was amplified using a specific designed primer set. The PCR product was evaluated by electrophoresis and then cloned in pET28a expression vector which transformed into E.coli BL21 (DE3) as a host. IPTG is used as an expression inducer in media. Alternatively, PCR products were analyzed by sequencing and double digestion with EcoRI and HindIII restriction endonuclease. The expressed protein was purified by Ni-NTA column and confirmed by SDS Page and western blotting. Evaluation of gene inhibition was carried out through Western blotting and RT-PCR.

Results: No mutation or sequence variants were found in PCR products as a result of sequencing analysis. Moreover, the quantity and quality of expressed recombinant protein in the presence of IPTG with selected vector in E. coli was high. VASH1 significantly prevented the expression of VEGFR2 mRNA expression and VEGFR2 protein. Evaluation of gene inhibition was carried out through western blotting and RT-PCR.

Conclusions: The produced vasohibin1 protein probably can be used as an angiogenesis inhibitor in further studies on retinopathies.

Keywords: Angiogenesis, Vasohibin1, Cloning, Gene expression.

Introduction

Angiogenesis is the formation of new blood vessels from pre-existing ones. It plays a central role in the progression of various chronic inflammatory diseases, including diabetic retinopathy.1 So, inhibition of angiogenesis may provide a rational approach to manage these diseases.

Induction of vascular endothelial growth factor (VEGF) by hypoxia triggers both physiological and pathological angiogenesis.2 VEGF plays a major role in the development of choroidal neovascularization.3 Therefore, VEGF inhibition has received the most attention in relation to potential anti-angiogenesis/anti-tumor therapy. Negative feedback regulation is one of the most important physiological mechanisms with which bodies are endowed, and has been demonstrated to control a wide range of phenomena.4

VEGF family members function through the activation of VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2), which are particularly expressed on vascular endothelial cells (ECs), and VEGF receptor-3 (VEGFR-3), which is restricted to lymphatic endothelium.5 In stimulating EC proliferation and migration, VEGFR-2 is more effective than VEGFR-1,6 and is markedly expressed during tumor growth, chronic inflammation, and wound repair.7

Vasohibin1 (VASH1) is induced by VEGF mediated by protein kinase C- δ downstream of VEGFR-2.8 It is a protein with 42 KD and 365 amino acids which can produce in different types of cells. The mode of VASH secretion is unconventional since VASH does not have a classical signal sequence.9 It inhibits migration and proliferation of endothelial cells in culture and exhibits a potent antiangiogenic activity in vivo.10 It might be deposed in the extracellular matrix (ECM) or be released into the blood to inhibit angiogenic processes of ECs.11 VASH1 can reduce VEGF-2 mRNA expression and then block VEGF-A (Hosaka et al., 2009). Thus, it has been assumed that VASH1 functions as a negative feedback inhibitor of angiogenesis and might be a promising candidate structure for antiangiogenic therapies.11-12

In our previous study, we reduced expression of VEGFR-1 and VEGFR-2.14-17 At present, VEGF is an important target to control vascular growth in vivo. The aim of this study was to clone and express VASH1 protein in order to inhibit the expression of VEGFR-2 in vitro for investigation of its antineovascularization effects in future studies.

Materials and Methods

The materials used and their sources were as follows: LB medium, LB agar, E. coli (DE3), kanamycin antibiotic, and VEGF165 were purchased from Sigma Co. pET28a(+) Plasmid was obtained from vector bank of the Pasteur Institute, Iran. The restriction enzymes including FastDigest EcoRI and FastDigest HindIII, Taq polymerase and T4 ligase, were
purchased from Fermentas Co. TAE, TE buffer, CTAB/NaCl buffer, agarose Gel SDS and phenol were purchased from Sigma, Roche and Fermentase Co. RNAs were isolated using RNeasy Mini kit (QiAGEN, Hilden, Germany). RT-PCR was performed using RNA PCR kit (AMV) (version 3.0; Takara Bio, Otsu, Japan).

The following primers were designed with Oligo primer analysis software based on the VASH1 gene sequence published in NCBI.


Additionally, all restriction enzyme cutting sites among the gene sequence were analyzed by DNA Star software. The specificity of designed primers was checked by aligning their sequence with the subjected sequence available in BLAST.

HUVECs as a source of VASH1 were cultured in PRMI 1640 medium and then stimulated with hypoxia and VEGF to induce the expression of VASH1 mRNA. Total RNA was extracted by RNA extraction kit. The cDNA was then synthesized by RT-PCR and amplified using PCR and specific primers. In this stage, Tag sequences containing recognition sites for restriction endonuclease EcoR I and HindIII were introduced into both ends of the gene. PCR product was evaluated by electrophoresis on 1% agarose gel.

Pure PCR products and pET28a vector were digested with EcoRI and HindIII restriction endonucleases, and then ligated with T4 DNA ligase followed by transforming into E. coli BL21 (DE3) using electroporation (Biorad, USA). After adding 1 ml SOC medium, they were placed in a 2 ml microtube and incubated at 37 °C for 2 h.

Transformed cells were centrifuged at max speed. Cell pellets were cultured in LB-Agar medium containing 40 µg/ml kanamycin at 37 °C for 24 h. Colonies able to grow in the presence of kanamycin were selected. In order to confirm cloning, PCR was performed on the vector as template using specific primers. A PCR product with 1098 bp in length indicates gene cloning in the vector.

With regard to the aim of the study; the expression of protein for use in future studies, evaluation of possible mutation was necessary. Following cloning and amplification of the cloned vector and prior to transforming into the host, the gene was sequenced by Macrogen (Korea) Company.

Colonies of 50 µl with positive PCR were added to 5 ml LB-broth containing 25 µl kanamycin and incubated at 37 °C for 2 h on a rotary shaker (150 rpm) to reach OD 0.6 at 600 nm. After adding 50 µl isopropyl-β-D-thiogalactoside (IPTG), the cells were incubated at 37 °C for 15 h on a rotary shaker (150 rpm). The cells were harvested and lysed in PBS pH 7.6 by high-pressure homogenizer. Afterward, cells were centrifuged at 4 °C (20 min. 12,000 g). The isolated inclusion bodies were washed twice with Tris solution (1.21 g per 100 ml distilled water) containing 0.186 g EDTA, 0.077g DTT at PH7 for the first time, and then containing 0.186 g EDTA, 0.077g DTT and 6 M urea the second time. Finally, the inclusion bodies were dissolved in 5 ml extraction buffer containing 8 M urea and dialyzed overnight at 4 °C to remove urea and produce proper folding in the protein.

His-tagged recombinant protein was added to NI column (16 * 125 mm), and then removed by adding 250 mM imidazol. The success of the purification was confirmed by SDS-PAGE electrophoresis and western blot analysis using antihistidine antibody.

To determine the effects of VASH1 on VEGFR-2, HUVECs were serum-starved overnight and incubated with purified recombinant VASH1 for 90 min, followed by 50 ng/mL VEGF165 treatment for 10 min. Cells were lysed with buffer containing 1% Triton X-100, 1% deoxycholate and proteinase inhibitor cocktail (Sigma). After centrifuge at 5,000 g, supernatant was used for western blotting, according to the Saito et al.18

HUVECs grown to 50% confluence were incubated either without or with VASH1 at appropriate concentrations for 3 days. The cells were then lysed and their total RNAs were isolated using RNeasy mini kit (QiAGEN, Hilden, Germany), according to the manufacturer’s instructions. RT-PCR was performed using RNA PCR kit (AMV) (version 3.0; Takara Bio, Otsu, Japan). Briefly, total RNAs were converted to cDNA at 50 °C for 30 min with reverse transcriptase and oligo-dT primer. Reverse transcription mixtures were subjected to PCR with specific primers. Reactions were incubated at 94 °C for 2 min and then were amplified using temperature parameters of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Amplifications were carried out for 35 cycles, followed by a 7-min extension at 72 °C. The products were separated by 1.5% agarose gel electrophoresis staining with 1 μg/ml ethidium bromide and visualized with a UV transilluminator.18

Results

After design and synthesis of specific primers for VASH1, the first total RNA was extracted from HUVECs. Then cDNA was synthesized by RT-PCR. By using specific primers, PCR product size of 1,098 bp was obtained (Figure 1).

Colonies of 50 µl with positive PCR were added to 5 ml LB-broth containing 25 µl kanamycin and incubated at 37 °C for 2 h on a rotary shaker (150 rpm) to reach OD 0.6 at 600 nm. After adding 50 µl isopropyl-β-D-thiogalactoside (IPTG), the cells were incubated at 37 °C for 15 h on a rotary shaker (150 rpm). The cells were harvested and lysed in PBS pH 7.6 by high-pressure homogenizer. Afterward, cells were centrifuged at 4 °C (20 min. 12,000 g). The isolated inclusion bodies were washed twice with Tris solution (1.21 g per 100 ml distilled water) containing 0.186 g EDTA, 0.077g DTT at PH7 for the first time, and then containing 0.186 g EDTA, 0.077g DTT and 6 M urea the second time. Finally, the inclusion bodies were dissolved in 5 ml extraction buffer containing 8 M urea and dialyzed overnight at 4 °C to remove urea and produce proper folding in the protein.

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Figure 1. 1098 bp product of vasohibin1- specific primers with 1098 bp length (Lane 1), Negative control without any cDNA (Lane 2) and High range DNA Ladder (Lane 3)
enzyme produced three fragments: 1,270 bp; 3,030 bp; 4,261 bp. The vector was also digested by EcorI and HindIII (Figure 2).

After vector confirmation using the restriction enzyme EcorI and HindIII, PCR product and vector were digested and ligated together with ligase followed by transforming into E. coli. Growing colonies in LB-Agar medium containing kanamycin were purified and the cloned gene was analyzed by PCR. The 1,098 bp band indicates cloning accuracy (Figure 3).

The cloned vector was also digested and the bands related to gene and vector body confirmed them (Figure 4). To evaluate mutation in the cloned gene, the fragments were sequenced and the results did not represent any non-sense mutation among the sequence (Figure 5).

After confirming cloning, vector was transformed into the host and the cells were cultured with or without IPTG. The cells were harvested and lysed to make the cellular extract. After
electrophoresis, the results were shown the expression of protein with IPTG in transfected cells (Figure 6). Recombinant protein was purified by Ni chromatography column (Figure 7) and was analyzed by using antihistidine antibody and western blotting (Figure 8).

**Discussion**

We have successfully cloned and expressed VASH1 as a recombinant in the prokaryotic host cell. The expression of proteins in E. coli is most widely and routinely used. VASH1 has been identified as a target gene of VEGF in human umbilical vein endothelial cells. The VASH negative feedback loop that modulates VEGF activity involves reduction of mRNA for VEGF-2. This can be achieved by inhibiting expression or by enhancing degradation of VEGF-2 RNA. The administration of exogenous VASH1 strongly inhibits pathological and physiological angiogenesis without any significant side effects. Furthermore, bolstering endogenous levels of VASH by injecting recombinant VASH1 or by administration of an adenoviral vector containing a vasohibin expression cassette, significantly suppressed retinal neovascularization.

The pET expression system is one of the common systems of cloning and expression of recombinant proteins in E. coli with specific properties including T7Lac promoter and 6 His Taq marker. The E.coli strains H5a (DE3) plysS, DH5a (DE3), BL21 (DE3) plysS, and BL21 (DE3) with lambda phage are able to express viral RNA polymerase. In the present study, the VASH1 gene was cloned into pET28a expression vector and transformed into E. coli BL21 (DE3), where it was induced to express VASH1 protein by IPTG. With careful choice of host strains, vectors, and growth conditions, most recombinant proteins can be cloned and expressed at high levels in E. coli. However, many polypeptide gene products expressed in E. coli accumulate as insoluble aggregates that lack functional activity. So, a primary consideration for recombinant protein expression and purification is the experimental purpose for which the protein will be utilized. For biochemical and structural studies, it is often important to optimize conditions for the expression of soluble, functionally active protein, whereas for antigen production, the protein can be expressed either in native or denatured form. The expressed protein migrated on SDS-PAGE at a position consistent with its molecular weight 45 KD. The number of bases which added to the cloned gene through ATG initiation codon, was 102 bases (34 amino acids) to add 3740 Da to the VASH1 molecular weight. This increase was the least of additional polypeptides in comparison to other studies. It can be cleaved by thrombin protein to enhance protein properties.

Human VASH1 gene was properly cloned in pET28a expression vector. The VASH1 recombinant protein was expressed in E. coli with proper concentration of IPTG. So, this recombinant system is a desired system for expressing this protein as soluble form. This work will definitely help those researchers who are all working with VASH1 worldwide and also with our future studies on VASH1.

**Acknowledgment**

This study was supported by the Baghiyallah University of Medical Sciences (TUMS), Tehran, Iran.
The authors thank the Chemical Injury Research Center for financial support and other colleagues in the Nanotechnology Research Center for scientific and research support.

**Conflict to Interest**

The authors declared that they have no conflict of interest.

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