Iranian Journal of Basic Medical Sciences

ijbms.mums.ac.ir

IJBMS

Heterologous expression of a truncated form of human recombinant vascular endothelial growth factor-A and its biological activity in wound healing

Mohsen Khaki [1], Ali Hatef Salmanian [2], Ghasem Mosayebi [1], Maryam Baazm [3], Saeed Babaei [3], Neda Molaee [1], Hamid Abtahi [1*]

[1] Molecular and Medicine Research Center, Department of Immunology and Microbiology, School of Medicine, Arak University of Medical Sciences, Arak, Iran
[2] National Institute for Genetic Engineering and Biotechnology, Tehran, Iran
[3] Department of Anatomy, School of Medicine, Arak University of Medical Sciences, Arak. Iran

ARTICLE INFO

Article type:
Original article

Article history:
Received: Feb 1, 2017
Accepted: May 25, 2017

Keywords:
Biological activity
Recombinant
VEGF-A
Wound healing

ABSTRACT

Objective(s): Vascular endothelial growth factor (VEGF) is one of the most effective proteins in angiogenesis, mesenchymal stem cells (MSCs) differentiation and wound healing. These abilities are therapeutic potential of VEGF in diabetic retinopathy, nephropathy and other tissue damage circumstances. In this study, recombinant VEGF was produced in *Escherichia coli* (*E. coli*) system and then biological activity of this protein was evaluated in animal wound healing.

Materials and Methods: *E. coli* BL21 (DE3) competent cells were transformed with pET32a-VEGF clone and induced by isopropyl-β-D-thio-galactoside (IPTG). The recombinant protein was purified by affinity chromatography. Recombinant VEGF-A-based ointment (VEGF/Vaseline 0.8 mg/100 w/w) was used for external wound (25×15mm thickness) healing in animal model. *In vivo* activity of ointment was evaluated by clinical evidences and cytological microscopic assessment.

Results: The recombinant protein with molecular weight of 45 kilodaltons (kDa) and concentration of 0.8 mg/ml was produced. Immunoblotting data showed that the antigenic region of VEGF can be expressed in *E. coli* and the recombinant protein has similar epitopes with close antigenic properties to the natural form. Macroscopic findings and microscopic data showed that the recombinant VEGF-A ointment was effective on excisional wound healing.

Conclusion: Recombinant VEGF-A produced by pET32a in *E. coli*, possesses acceptable structure and has wound healing capability.

► *Please cite this article as:*
Khaki M, Salmanian AH, Mosayebi Gh, Baazm M, Babaei S, Molaee N, Abtahi H. Heterologous expression of a truncated form of human recombinant vascular endothelial growth factor-A and its biological activity in wound healing. Iran J Basic Med Sci 2017; 20:791-797. doi: 10.22038/IJBMS.2017.9011

Introduction

Vascular endothelial growth factor-A (VEGF-A) is a dimeric protein made up of two identical monomer subunits. Each monomer chain contains a site for N-linked glycosylation, but the glycosylation is not required for its *in vitro* or *in vivo* activity. The gene encoding VEGF consists of eight exons, which directs the expression of seven different versions of the VEGF (1). It is an endothelial cell-specific mitogen that is produced by many cell types including tumor cells, macrophages, platelets, keratinocytes and renal mesangial cells (2). Additionally, VEGF signals through tyrosine kinase receptors VEGFR1/flt-1, VEGFR2/flk-1 and VEGFR3/flt-4 (Fms-Related Tyrosine Kinase). It also binds neuropilin co-receptors (NRP-1 and NRP-2). Presently, VEGF and its receptors are major targets for several cancer therapies (3-5).

VEGF plays several roles in normal physiological functions and pathological situations. VEGF-A facilitates endothelial cells (EC) proliferation, migration and recruitment, and generally participates in the early phase of blood vessel formation by vasculogenesis, angiogenesis and wound healing (3). At *in vitro* condition, mesenchymal stem cells (MSCs) differentiate into ECs in the presence of VEGF-A. Application of VEGF-A and MSC-derived ECs at the interventional site is a complex clinical challenge (6-8). Functional superiority of the VEGF has been shown in endothelial cell migration and proliferation and ultimately, in the formation of arterial and venous systems (3, 4, 9, 10).

As VEGF has multiple applications and this protein is produced in small amounts naturally, mass production methods for VEGF that provide high yield as well as high purity, quality and potency is highly required (5, 11).

*Corresponding author: Hamid Abtahi, Molecular and Medicine Research Center, Arak University of Medical Sciences, Arak, Iran. Tel: +98- 861-4173502; email: abtahi@arakmu.ac.ir

According to the above mentioned about VEGF and glycosylation, eukaryotic expression systems are not mandatory to produce an active, therapeutic and effective form of VEGF. And bacterial expression systems such as *Escherichia coli* (*E. coli*) are accessible, economical, and cost-effective, which provide controllable alternative host cells with a high potency for production, extraction and purification of recombinant VEGF-A(11). In this regard, heparin binding region of VEGF-A was expressed in Chinese hamster ovary cells (CHO) or *E. coli* K12 strain by pET14b vector, separately. Advantages and disadvantages of these systems are discussed subsequently (2, 12).

In this study, expression of the VEGF-A was carried out in *E. coli* (BL21 DE3) competent cell, with pET32a expression vector, as an accessible and cost-effective microbial expression system. To assay the biological activity of the recombinant VEGF protein, the animal excisional wound healing model was applied for evaluating VEGF in cutaneous healing and repair.

Materials and Methods

Gene, vector and cells

The sequence of VEGF-A165 (Acc. NM_001287044) contains BamHI excision site on its 5′ position and excision site for XhoI on its 3′ end (Biomatic.Co. Canada). The gene was cloned in pSK plasmid. *E. coli* DH5α (Stratagene, La Jolla, Calif) as the primary host cell and *E. coli* BL21 (DE3) and *E. coli* BL21 (DE3) pLysS (Novagen, USA) as competent cells were used.

Gene cloning

The pSK-VEGF was transformed into *E. coli* DH5α. The pSk-VEGF purification was performed by plasmid mini prep kit (Qiagen, Hilden, Germany). The pSk-VEGF was double digested by restriction enzymes (BamH1, Xho1: Roche, Penzberg, Germany) and ligated by DNA T4 Ligase (Cinaclone, Tehran, Iran). Ligated product was transformed into *E. coli* DH5α.

The pET32a-VEGF was transformed into expression host. Bacterial cells were grown in nutrient broth supplemented with 100 µg/ml ampicillin and ampicillin plus chloramphenicol 100 µg/ml, respectively at 37 °C with agitation. For production of recombinant protein, we used LB broth enrichment by 10 g NaCl, 1 g KCl, 0.5 g $MgCl_2$, 0.5 g $CaCl_2$, 14 g yeast extract, and 12 g Bactotryptone. The protein induction was performed in media using final concentration of 1 mM isopropyl-β-D-thio-galactoside (IPTG) (Thermo Scientific, Italy). Cultures incubated at 37 °C and vigorously agitated at 200 rpm (optical density reached 0.6 at 600 nm).

Protein purification

According to SDS-PAGE results, protein purification of DE3 was performed just 2 hr after sample induction. Since there are 6 His.tag linked to protein by pET32a, the expressed protein was purified using Ni-NTA column (Qiagen, CA, USA).

Protein dialysis

The purified protein was dialyzed using manual procedure (13) and trade specific device (Slide-A-Lyzer Dialysis Cassettes, Pierce, Rockford, USA) with membrane molecular- weight cut off of 3.5 kDa against phosphate buffered saline (PBS) (Merck, Germany), pH 7.7 at 22 °C/4 hr and 4 °C/overnight. ExPASy site (www.expasy.org) was used for determination of protein pI and pH.

The quality of the purified recombinant proteins was assessed by SDS-PAGE (12%) and the quantity was determined with the absorbance of 280 and 260 nm. Proteins concentration was measured using Bradford protein assay.

Immunoblot analysis of recombinant VEGF

Immunoblot assay was performed using 0.5 µg of purified recombinant protein per well. After SDS-PAGE, the gel was blotted on to polyvinylidene difluoride (PVDF) membrane, (Roche Molecular Biochemicals, Mannheim, Germany) using 25 mM Tris buffer, pH 8.3 as transfer buffer, 192 mM glycine and 20% methanol at 90 volts for 1.5 hr at 4 °C. The blotted membrane was blocked with 1% (w/v) skim milk (Roche, Mannheim, Germany). Primary Ag-Ab reaction was performed using specific anti serum human-anti-VEGF (abcam-ab1316 Cambridge. UK). The blots were washed with Tris Buffered Saline with Tween 20(TBST, Roche, Mannheim, Germany). Membrane was treated with conjugated anti human IgG horseradish peroxidase (HRP) (Abcam, Cambridge, UK). Reaction was developed by diamino-banzidine (DAB) solution (Abcam, Cambridge, UK) in dark condition.

Animal model excisional wound healing

This study was approved by the internal animal ethics committee of the Molecular and Medicine Research Center, Arak University of Medical Sciences, Arak, IR Iran (ethical code R, ARAKMU.REC.1394.199) in accordance with Portuguese law.

Male Wistar rats (250-300 g and 8-10 week) were purchased from Pasteur Institute of Iran. Animals kept in suitable animal room with temperature 20-25 °C, humidity 50%-70% and a 12:12 hr light/ dark cycle and were housed in groups of 3. Rats were alimented by specific rodent pellet and urban tap water. In this study, 18 rats were divided into 3 groups equally (14).

Case group: Treated with recombinant VEGF-based ointment: VEGF/Vaseline (Anglo-Dutch Company. USA): 0.8 mg/100 w/w

Control group: No drug treated (naturally healing).

Sham group: Treated with just Vaseline.

Animals were anesthetized with 50 mg/kg ketamine (Rotexmedica. Germany) and 5 mg/kg xylazine (Alfasan. Holland). The panniculus carnosus muscle shaved area was selected for 25 mm×15 mm

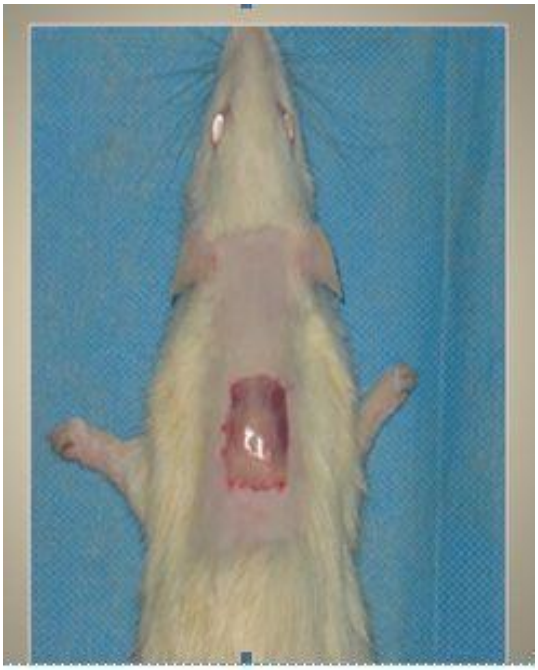

Figure 1. The rat panniculus carnosus muscle excisional wound on the 0th day

thickness excisional wound (Figure 1). Wound was not sutured or covered (15).

Drug treatment

Case group was treated with recombinant VEGF-A based ointment locally and cutaneously at a dose of 5 g ointment per each rat once a day. Control group were allowed to treat without intervention (naturally healing) and sham group were treated with Vaseline. Administration was continued for 7 days.

Wound area (unit: mm2) was measured by Caliper (precision rate: 0.1 mm) on days 3, 5 and 7 for macroscopic changes. For microscopic analysis, on days 3, 5 and 7, rats were sacrificed by high inhalation dose of ether. Wound area tissues were fixed in formalin (pH 6.9). Embedded paraffinized samples were used for sectioning by stereological technique. Cytological criteria such as neovascular formation, and epithelium thickness were evaluated in hematoxylin and eosin (H&E) stained 6 µm thickness section by stereological method (16). (Figure 2).

Statistical analysis

The experimental results of animal study were analyzed by independent sample t-tests to evaluate variables in each group and the results were compared using the Mann–Whitney U-test. All data were analyzed using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA) and expressed as mean±SD. *P-values*<0.05 were considered statistically significant.

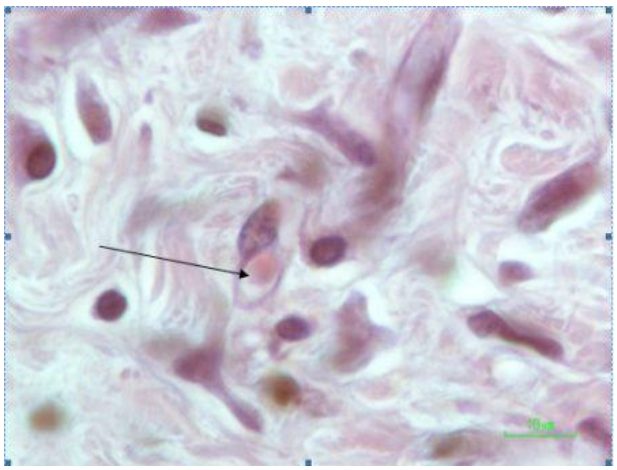

Figure 2. Granulation tissue and neovascular view. The 5 mm sections were stained with hematoxylin and eosin (H&E) and observed by 10-100 objective lenses (NIKON Eclipse E2000-Videocamera DS-Fi1, Japan). Blood vessels and neovasculars were identified by the presence of blood cells in their lumens (Arrow notes the neovascular)

Results

The pSK-VEGF was digested with BamH1 and XhoI restriction enzymes. As it is shown in Figures 3 and 4, IPTG induction process showed that the gene expression and protein production in DE3 2 hr after induction is considerably better than the gene expression in *E.coli* pLysS cells, in different time points after induction. The results of protein extraction by Ni-NTA-Agarose kit showed that the yield of protein was higher in DE3 cell than in pLysS cell (0.8 and 0.1 mg, respectively).

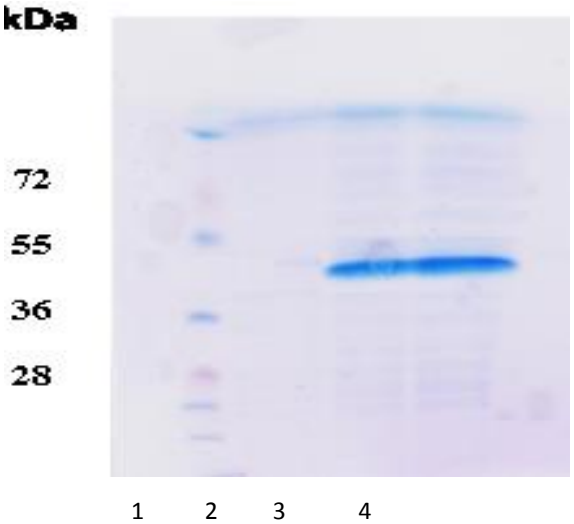

Figure 3. Protein induction by isopropyl-β-D-thio-galactoside (IPTG) (Line 1: Marker, Line 2: Before induction, Line 3 and 4: After induction)

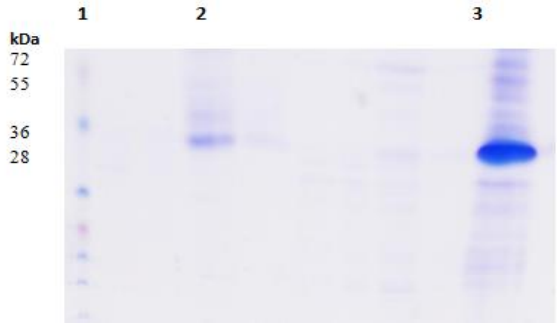

Figure 4. Approved purified proteins in pLysS and *E. coli* BL21 (DE3) (Line 1: Marker, Line 2: pLysS, Line 3:DE3)

Data of protein dialysis using PBS buffer indicated that the best band on PAGE was observed after 24 hr shaking vigorously in 4^{0}C and pH 8 (Figure 5).

As shown in Figure 6, western-blot analysis confirmed the specific immunoblotting interaction between the recombinant VEGF-A and specific antibody (Antibody VG-1ab1316)

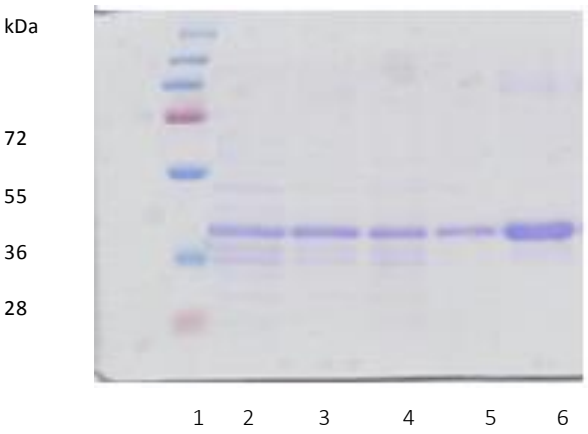

Figure 5. SDS-PAGE of vascular endothelial growth factor (VEGF) protein analysis
Line 1: Marker, Line 2: pH 6 Line 3: pH 6.5, Line4: pH 7, line 5: pH 7.5, line 6: pH 8

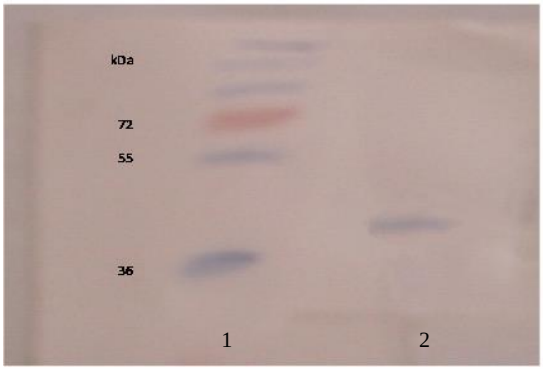

Figure 6. Western-blot assessment
Line 1: Marker, Line 2: Positive Ag-Ab reactivity

Results of VEGF activity in cutaneous wound healing

Animal study showed that the healing criteria of the case group was higher than both sham and control groups. Control group mice had similar tissue properties as the sham group, with no apparent differences in the neovascularization and wound area. At 3, 5 and 7 days post wounding, case group animals had a considerable progressive wound healing. At day 7, the mean wound area of case group was 75 mm^2 (SD: 5.50), while the mean wound area in control and sham group was 153 (SD: 11.93) and 146 (SD: 5.68) mm^2, respectively. Data analysis by *post- hoc* Tukey's honest significant difference (HSD) test showed that there was significant difference between case group with sham and control groups in wound area and epithelium thickness at 3, 5 and 7 days (*P*: 0.00- 0.04). At 7th day, the mean neovascular counts of case, control and sham groups were 61, 43 and 45, respectively (SD: 9.60, 4.70 and 4.16, respectively). On days 1 to 5, although the neovascular count in the case group was more than control groups, the differences were not significant (*P*: 0.95- 0.38). Results were presented in Figures 7 and 8.

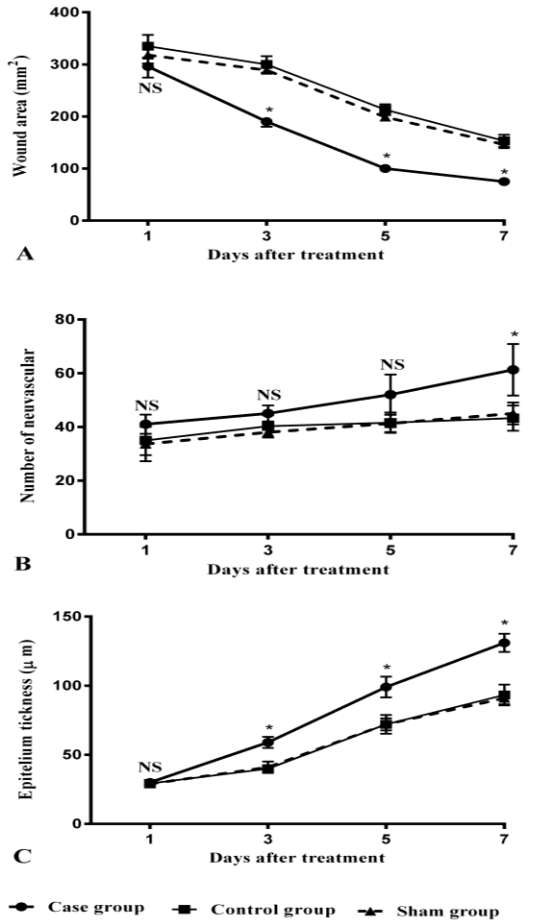

Figure 7. Effects of vascular endothelial growth factor (VEGF) treatment on wound healing. (A) Measurement of the wound area after treatment. (B) Number of neovascular after treatment. (C) Epithelium thickness after treatment. NS: not significant, *: significant

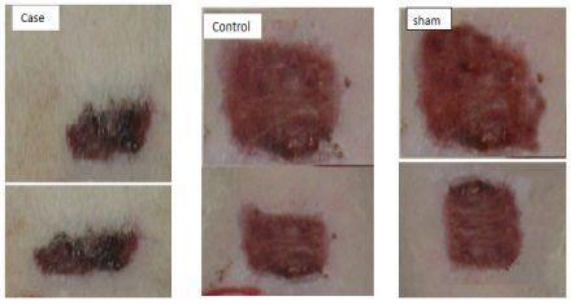

Figure 8. Pictures of mice wound area at 7th day post wounding

Results of microscopic assessment showed that granulation tissue formation and its tissue expanding in case group was less than in control and sham groups. On the other hand, tissue contraction of lesion in case group was more than other groups. Lack of wound contraction in control and sham groups is coordinated with less healing tissue indexes including fibroblast and blood vessels (Figure 9).

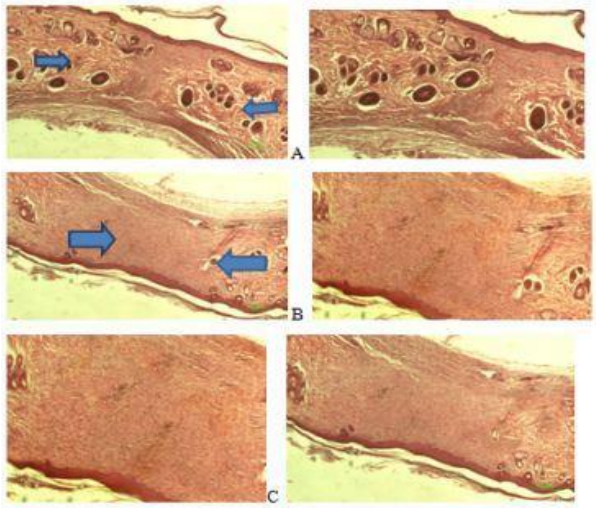

Figure 9. Microscopic tissue area on the 7th day of healing course shows evidence of wound contraction with healing tissue (fibroblasts and blood vessel proliferation). Arrow notes the wound's surface and neovascular. (A): case, (B): control, (C): sham

Discussion

Multiple applications of recombinant proteins in medicine, agriculture and industry have led to an increase in requests for the use of bacteria, fungi, plants, and insects as accessible expression systems. Bacterial expression systems have become the most popular hosts in this process. This is also confirmed by the increasing number of studies on strains of bacteria (5, 11, 12).

In this study, the bacterial expression cells and other materials such as vectors have been shown to be suitable for production of recombinant VEGF. Results indicated that the yield of recombinant VEGF was higher in DE3 cells than in *E. coli* (BL21) pLysS. Protein expressions in DE3 and DE3 pLysS are induced by IPTG. Based on the results of the current study, recombinant VEGF is produced at higher level in the DE3 cells than in pLysS cells because DE3 is one of the most efficient expressive hosts that contains T7 RNA polymerase, which is controlled by the chromosomal Lac UV5 promoter and plasmid T7 lysozyme. Since the T7 lysozyme is coded in the pLysS cells, it is probable that a considerable amount of cell energy is consumed during this process. This event might explain why the amount of the produced protein is lower in DE3 pLysS cells. On the other hand, the produced T7 lysozyme in pLysS cells reduces T7 RNA polymerase, and subsequently it reduces protein production yield (12, 17).

The results showed that the process of protein absorption by resin and nickel had correctly occurred. Indeed, the yield of protein extraction based on the kit's instruction was not really considerable. Nevertheless, with some little modifications in the protocol, including prolonging the washing step with the wash buffer, reducing the time of sample exposure to resin and modifying the shaker speed and the tube size, remarkable improvement in the yield can be observed.

In this study, because of adding "His-tagged" extension to the N-terminal of the recombinant protein that was obtained from pET32a vector, extraction of the protein could be performed by affinity chromatography using Ni-NTA resin, whereas other vectors, such as pBR322, did not produce a tag to facilitate extraction of the recombinant protein. Additionally, the pET32a vector had a strong T7 promoter that facilitates high amount of gene transcription (18, 19).

The results of current study confirmed the successful refolding of the protein after dialysis, because the antigenic reactivity of the final product with specific antiserum can take place only when the physicochemical structure of the protein is remained intact. Based on these results, the recombinant VEGF was dialyzed against PBS and converted to the refolded and active protein. The presence of the protease enzyme in the host cell is one of the influential factors in the production of recombinant proteins. Since *E. coli* lacks the Lon and ompt proteases, it can be considered as a valid and practical expression cell for the production of recombinant proteins (17, 20-22).

In an experimental study, VEGF was produced using CHO cells, and since the host cells were eukaryotic, the protein glycosylation was performed on the product (2). Nevertheless, the current study has several differences with the mentioned study in which the eukaryotic cells have the advantage of glycosylation of the protein after translation. However, the glycosylation is not necessary for VEGF activities (2). In this process, due to the use of the bioreactor, there was a high protein yield. It is obvious that these systems have a higher efficiency for industrial level and mass-productions. Therefore,

under limited resources, this strategy is more applicable and economical.

Different studies have used variant pET vectors and different *E. coli* strains (12, 23) and some of the results are similar to or different from those of the current study. In one research, *E. coli* K12 Escherichia strain and pET14b as the vector were used for heparin binding region of VEGF A165 (VEGFA 165 HBD), which is different from the current study in terms of vector and the host. Furthermore, that study focused on a particular region of the VEGF (12).

In another study, high cell density fed-batch fermentation and three chromatography steps were applied for producing recombinant VEGF in bacterial expression system. The *E. coli* host strain and 64D1 and pBR322 plasmids were practically productive cell and vectors, respectively. The differences between this study and ours research were the type of vector and gene-expressing cells as well as the methods of protein purification. Protein refolding method was confirmed by mass spectrometry and sequencing, and then biological activity of VEGF was evaluated by the proliferation of endothelial cells. Taking advantage of this method depends on having multiple equipment including high affinity fermentation system, oxygen/nitrogen, monitoring, etc. Financial and logistical support for this equipment has rarely been performed for protein production in laboratory scale (23).

Data of animal model excisional wound healing confirmed the biological activity of recombinant VEGF. This protein accelerated cutaneous tissue replacement and promoted clinical and cytological criteria of dermal healing and repair.

Complete wound healing happened within less than 10 days in the case group. Short time wound healing process confirmed that the recombinant refolded VEGF protein was active in the wound healing process (24, 25).

Control and sham groups were close in macroscopic and stereological findings; this means that natural healing and Vaseline treatment had the same results.

Based on the results of this study, considering the role of natural VEGF as a pleiotropic growth factor in tissue healing and its role in multiple process, including neovascularization, re-epithelialization, and regulation of collagen formation,

it seems that the recombinant protein, like the natural VEGF, are involved in the complex processes of wound healing.

From the third day, the amount of epithelium thickness and wound area in case group was significantly different from the control and sham groups, but in the neovascularization, significant difference between the groups was observed from the seventh day. In other words, in wound healing, angiogenesis is a delayed process induced by the recombinant synthetic VEGF.

Keswani *et al.* hypothesized that salivary VEGF is an essential growth factor for healing oral mucosal tissue. The sialoadenectomized murine was applied as a model without VEGF in salivary glands. They applied only VEGF for wound healing orally. Their results indicated that the salivary-VEGF had a novel role in mucosal wound healing and they provided a scientific hypothesis for the improvement of ideal therapeutic procedures. In this study, the role of salivary-VEGF in the process of wound healing was evaluated and the effectiveness of clinical factors and angiogenic protein was measured. But unlike the present study, in their study, the used VEGF was not a recombinant protein (26).

Nauta *et al.* concluded that commercially transfection reagent (plasmid DNA encoding VEGF) led to VEGF overexpressing and accelerated wound healing. They proposed that their molecular system can be used for treating wound healing as an alternative therapy (27).

In other studies, insulin like growth factor (IGF-1) linked to adenoviral vector, an artificial molecular device, was used. They hypothesized that expression of this system will increase wound healing by inducing angiogenesis via VEGF dependent pathway. Results of this study showed that the overexpression of this genomic compartment increased capillary density and keratinocyte migration and promoted some other related wound healing criteria (28, 29).

Further investigation has shown that intramuscular administration of recombinant human-VEGF-A gene to patients with peripheral arterial disease and ischemic ulcers resulted in limb salvage to significantly decrease rest pain (30). Furthermore, gene therapy and direct application of VEGF is effective in healing and repair of wounds.

Conclusion

The results of the current study showed that using DH5α strain of *E. coli* for early transformation and using *E. coli* (BL21 DE3) as host and pET32 as vector could produce VEGFA165 that is one of the most demanded proteins for medical needs in laboratory and industrial levels. In conclusion, our data in manufactured recombinant VEGF-based ointment confirmed that homemade recombinant VEGF can be used as a potential alternative therapeutic agent in multiple drug forms.

Acknowledgment

This study is a part of the thesis proposal of PhD by research (N: 2356, ethical code R, ARAKMU. REC.1394.199, Arak University of Medical Sciences). This study was conducted with financial assistance from Molecular and Medicine Research Center, Arak University of Medical Sciences, Arak, Iran, and we are grateful for their invaluable contribution to this

study. The authors would like to thank the Deputy of Research and Technology of Arak University of Medical Sciences and educational and research specialists of Arak University of Medical Sciences for equipment support.

References

1. Vempati P, Popel AS, Mac Gabhann F. Extracellular regulation of VEGF: isoforms, proteolysis, and vascular patterning. Cytokine Growth Factor Rev 2014; 25:1-19.
2. Lee SB, Park JS, Lee S, Park J, Yu S, Kim H, *et al.* Overproduction of recombinant human VEGF (vascular endothelial growth factor) in Chinese hamster ovary cells. J Microbiol Biotechnol 2008; 18:183-187.
3. Berendsen AD, Olsen BR. How vascular endothelial growth factor-A (VEGF) regulates differentiation of mesenchymal stem cells. J Histochem Cytochem 2014; 62:103-108.
4. Cai Q, Brissova M, Reinert RB, Pan FC, Brahmachary P, Jeansson M, *et a*l. Enhanced expression of VEGF-A in beta cells increases endothelial cell number but impairs islet morphogenesis and beta cell proliferation. Dev Biol 2012; 367:40-54.
5. Makrides SC. Strategies for achieving high-level expression of genes in Escherichia coli. Microbiol Rev 1996; 60:512-538.
6. Hiley CT, Chard LS, Gangeswaran R, Tysome JR, Briat A, Lemoine NR, *et al*. Vascular endothelial growth factor A promotes vaccinia virus entry into host cells via activation of the Akt pathway. J Virol 2013; 87:2781-2790.
7. Igarashi Y, Chosa N, Sawada S, Kondo H, Yaegashi T, Ishisaki A. VEGF-C and TGF-beta reciprocally regulate mesenchymal stem cell commitment to differentiation into lymphatic endothelial or osteoblastic phenotypes. Int J Mol Med 2016; 37:1005-1013.
8. Li C, Zhen G, Chai Y, Xie L, Crane JL, Farber E, *et al*. RhoA determines lineage fate of mesenchymal stem cells by modulating CTGF-VEGF complex in extracellular matrix. Nat Commun 2016; 7:11455.
9. Ikhapoh IA, Pelham CJ, Agrawal DK. Atherogenic cytokines regulate VEGF-A-induced differentiation of bone marrow-derived mesenchymal stem cells into endothelial cells. Stem Cells Int 2015; 2015:498328.
10. Fernandez-Alonso R, Martin-Lopez M, Gonzalez-Cano L, Garcia S, Castrillo F, Diez-Prieto I, *et a*l. p73 is required for endothelial cell differentiation, migration and the formation of vascular networks regulating VEGF and TGFbeta signaling. Cell Death Differ 2015; 22:1287-1299.
11. Sorensen HP, Mortensen KK. Advanced genetic strategies for recombinant protein expression in Escherichia coli. J Biotechnol 2005; 115:113-128.
12. Seyedarabi A, Cheng L, Zachary I, Djordjevic S. Production of soluble human vascular endothelial growth factor VEGF-A165-heparin binding domain in Escherichia coli. PLoS One 2013; 8:e55690.
13. Moghadam M, Ganji A, Varasteh A, Falak R, Sankian M. Refolding process of cysteine-rich proteins:Chitinase as a model. Rep Biochem Mol Biol 2015; 4:19-24.
14. Legeza VI, Grebenyuk AN, Kondakov AY, Zargarova NI. [Comparative evaluation of healing wounds at a local and combined radiation injury in an experiment]. Radiats Biol Radioecol 2015; 55:584-590.
15. Galiano RD, Michaels Jt, Dobryansky M, Levine JP, Gurtner GC. Quantitative and reproducible murine model of excisional wound healing. Wound Repair Regen 2004; 12:485-492.
16. Stuart DA, Oorschot DE. Embedding, sectioning, immunocytochemical and stereological methods that optimise research on the lesioned adult rat spinal cord. J Neurosci Methods 1995; 61:5-14.
17. Sambrook J, Russell DW. Molecular cloning: a laboratory manual 3rd edition. UK: Coldspring-Harbour Laboratory Press; 2001.
18. Molaee N, Abtahi H, Mosayebi G. Expression of recombinant streptokinase from streptococcus pyogenes and its reaction with infected human and murine sera. Iran J Basic Med Sci 2013; 16:985-989.
19. Sadoogh Abbasian S, Ghaznavi Rad E, Akbari N, Zolfaghari MR, Pakzad I, Abtahi H. Overexpression and enzymatic assessment of antigenic fragments of hyaluronidase recombinant protein from streptococcus pyogenes. Jundishapur J Microbiol 2015; 8:e13653.
20. Claffey KP, Senger DR, Spiegelman BM. Structural requirements for dimerization, glycosylation, secretion, and biological function of VPF/VEGF. Biochim Biophys Acta 1995; 1246:1-9.
21. Sugimura K, Higashi N. A novel outer-membrane-associated protease in Escherichia coli. J Bacteriol 1988; 170:3650-3654.
22. Abtahi H, Salmanian AH, Rafati S, Behzadian Nejad G, Mohammad Hassan Z. High level expression of recombinant ribosomal protein (L7/L12) from Brucella abortus and its reaction with infected human sera. Iran Biomed J 2004; 8:13-18.
23. Pizarro SA, Gunson J, Field MJ, Dinges R, Khoo S, Dalal M, *et al*. High-yield expression of human vascular endothelial growth factor VEGF(165) in Escherichia coli and purification for therapeutic applications. Protein Expr Purif 2010; 72:184-193.
24. Mendes JJ, Leandro CI, Bonaparte DP, Pinto AL. A rat model of diabetic wound infection for the evaluation of topical antimicrobial therapies. Comp Med 2012; 62:37-48.
25. Scrofani SD, Fabri LJ, Xu P, Maccarone P, Nash AD. Purification and refolding of vascular endothelial growth factor-B. Protein Sci 2000; 9:2018-2025.
26. Keswani SG, Balaji S, Le LD, Leung A, Parvadia JK, Frischer J, *et al*. Role of salivary vascular endothelial growth factor (VEGF) in palatal mucosal wound healing. Wound Repair Regen 2013; 21:554-562.
27. Nauta A, Seidel C, Deveza L, Montoro D, Grova M, Ko SH, *et al*. Adipose-derived stromal cells overexpressing vascular endothelial growth factor accelerate mouse excisional wound healing. Mol Ther 2013; 21:445-455.
28. Balaji S, LeSaint M, Bhattacharya SS, Moles C, Dhamija Y, Kidd M, *et al.* Adenoviral-mediated gene transfer of insulin-like growth factor 1 enhances wound healing and induces angiogenesis. J Surg Res 2014; 190:367-377.
29. Basso FG, Pansani TN, Turrioni AP, Bagnato VS, Hebling J, de Souza Costa CA. *In vitro* wound healing improvement by low-level laser therapy application in cultured gingival fibroblasts. Int J Dent 2012; 2012:719452.
30. Kusumanto YH, van Weel V, Mulder NH, Smit AJ, van den Dungen JJ, Hooymans JM, *et a*l. Treatment with intramuscular vascular endothelial growth factor gene compared with placebo for patients with diabetes mellitus and critical limb ischemia: a double-blind randomized trial. Hum Gene Ther 2006; 17:683-691.