Original article

Mesenchymal Stem Cells Differentiate to Endothelial Cells Using Recombinant Vascular Endothelial Growth Factor –A

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Abstract

Background: Vascular endothelial growth factor-A (VEGF-A), an endothelial cell-specific mitogen produced by various cell types, plays important roles in cell differentiation and proliferation. In this study we investigated the effect of recombinant VEGF-A on differentiation of mesenchymal stem cells (MSCs) to endothelial cells (ECs).

Methods: VEGF-A was expressed in *E. coli* BL21 (DE3) and BL21 pLysS competent cells with the pET32a expression vector. Recombinant VEGF-A protein expression was verified by SDS-PAGE and western blotting. Mesenchymal stem cell differentiation to ECs in the presence of VEGF-A was evaluated by flow cytometry and fluorescence microscopy.

Results: Recombinant VEGF-A was produced in *E. coli* BL21 (DE3) cells at 0.8 mg/mL concentration. Expression of CD31 and CD 144 was significantly greater, while expression of CD90, CD73, and CD44 was significantly less, in MSCs treated with our recombinant VEGF-A than in those treated with the commercial protein ($p < 0.05$).

Conclusions: Recombinant VEGF-A expressed in a prokaryotic system can induce MSCs differentiation to ECs and can be used in research and likely therapeutic applications.

Keywords: Cell differentiation, Endothelial cell, Mesenchymal stem cell, Vascular Endothelial Growth Factor A

Introduction

Vascular endothelial growth factor (VEGF), also called vascular permeability factor (VPF), is an important angiogenic growth factor produced by multiple cell types including macrophages, platelets, tumor cells, keratinocytes, and others (1-4).

Vascular endothelial growth factor consists of two subunits, each of which contains N-linked glycosylation sites not necessary for its activity. VEGF is encoded by eight exons and expressed as seven different isoforms based on gene expression and exon splicing. Vascular endothelial growth factor-A 165, the major VEGF isoform, has been extensively studied and considered for potential paraclinical and therapeutic applications (4).

The critical roles of VEGF in endothelial cell migration, proliferation, and formation of the arterial and venous systems were evaluated formerly. Vascular endothelial growth factor was initially identified as an important factor in angiogenesis and vasculogenesis. Clinical studies confirmed that mesenchymal stem cells (MSCs), which are pluripotent cells, can differentiate into a variety of cells (5). In this regard, MSCs can differentiate into endothelial cells (ECs) in the

presence of VEGF-A, through which stem cell-based therapies have been proposed to support endothelial regeneration (6-9).

With respect to multiple applications of VEGF in therapeutic and interventional procedures, VEGF-A has been produced using both prokaryotic and eukaryotic cells and its activity has been evaluated (10-12). However, one VEGF domain has not been evaluated for angiogenesis or vasculogenesis (13, 14). Several attempts have been made to evaluate MSCs differentiation into ECs using commercial VEGF-A (1, 2); therefore, designing an accessible and cost-benefit system to mass produce VEGF-A and evaluate its role in MSC differentiation into EC is necessary.

We hypothesized that recombinant VEGF-A can induce MSC differentiation to endothelial cell; therefore, our aim in this study was to produce recombinant VEGF-A and analyze its ability to stimulate MSC differentiation. The microbial expression system described in this study is an accessible, practical, and inexpensive method of producing suitable amounts of recombinant VEGF-A for clinical and paraclinical procedures.

Materials and Methods
Recombinant protein cloning and expression
All procedures performed in this study were in accordance with the ethical standards of the Ethics Committee of Arak University of Medical Sciences (ARAKMU.REC.1394.199) (15). The VEGF-A protein-coding region was obtained from NCBI gene bank database (accession no. NM_001204384) and the gene construct was synthesized by Biomatic Company (Biomatic Corporation, Cambridge, Ontario). DH5α (Stratagene, La Jolla, Calif.) as a replicative host cell were transformed with the plasmid construct containing the VEGF-A gene (*VEGF-A*). Plasmids were purified using a QIA Prep Spin Miniprep Kit following the manufacturer's instructions (Qiagen, Hilden, Germany)**.** The target gene and pET32a (Novagen, Madison, USA) were double digested with restriction enzymes BamH1 and Xho1 (Roche, Penzberg, Germany) and ligated, and the ligated construct was used to transform *E. coli* BL21 (DE3) and BL21 (DE3) pLysS (National Institute for Genetic Engineering and Biotechnology, Tehran,

Iran) cells as the expression host cells at 37 °C. The proteins were induced using isopropyl-β-D-thio-galactoside (IPTG*;* Thermo Scientific, Italy*)* to a final concentration of 0.1μg/ml and incubated for more than eight hours. The bacterial cells were centrifuged at 1700 × g for 15 min. and the supernatant was collected for protein purification.

Recombinant protein purification and validation
Expressed proteins were purified on a Ni-NTA column (Qiagen, CA, USA) and dialyzed using dialysis cassettes (Pierce, Rockford, USA) with a molecular-weight cut off (MWCO) of 3.5 kDa against PBS, (pH 7.7) at 22 °C for 2 hours, followed by incubation at 4 °C overnight. The pI and pH of the protein were determined using the ExPASy site (www.expasy.org). The purified recombinant proteins were separated on a reducing 12.5% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE; Bio-Rad, CA, USA) and the proteins were quantified by Bradford assay (Bio-Rad, Hercules, USA) (16).

Western blots
Expression of the purified recombinant proteins was analyzed by western blotting. After SDS-PAGE, the separated proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Roche Diagnostics, Mannheim, Germany). The membrane was blocked with 1% (w/v) skim milk (Roche, Mannheim, Germany), and incubated with 1:500 dilution of primary anti-VEGF (Abcam, Cambridge. UK) in 2% bovine serum albumin (BSA) overnight at 4 °C. After washing in PBS, the membrane was incubated with a 1:5000 dilution of horseradish peroxidase-(HRP) conjugated anti-human antibody (Abcam, Cambridge, UK). The blots were developed with diaminobanzidine (DAB) solution (Abcam, Cambridge, UK) in the dark.

MSC culture and differentiation
Mesenchymal stem cells (Pasteur Institute, Tehran, Iran) were cultured at 37 °C in 95% humidity and 5% CO2. The cell cultures were grown in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Paisley, UK) supplemented

with 2 mM L-glutamine (Gibco, NY, USA) and 10% fetal bovine serum (FBS; Gibco, NY, USA). For MSCs differentiation, 50 ng/mL of commercial VEGF-A (Abcam, MA, USA) or synthetic recombinant VEGF-A were added to the culture media at 50% confluency for 10 days and the media was changed every 48 hours. On the day of the experiment, the medium was discarded and 5×10^4 cells/well were treated in duplicate with PBS as the negative control, commercial recombinant human VEGF-A as the positive control, and synthetic recombinant human VEGF-A. At the end of the 10th day of differentiation, the cells were dislodged from the culture plates with PBS containing 4 mM EDTA (Sigma-Aldrich, Munich, Germany). The cells were then washed with PBS and analyzed by flow cytometry.

Flow cytometry

At the end of the 10-day differentiation protocol, MSCs and ECs were evaluated by three color-flow cytometry using a FACSCalibur flow cytometer (BD Biosciences, CA, and USA). For this purpose, 2×10^6 MSCs and ECs/mL were incubated for 60 min at 4 °C with FITC-conjugated anti-CD31 and anti-CD34 and phycoerythrin (PE)-conjugated anti-CD90, anti-CD105, and anti-CD144, and PerCP-conjugated anti-CD44 and anti-CD73, separately. PerCP-conjugated mouse IgG1 or IgG2a were used as isotype controls. All the monoclonal antibodies were purchased from Abcam (Abcam, MA, USA).

Fluorescence microscopy

Mesenchymal stem cells cultures were prepared in separate slide flasks (Nunc, Nuncolon, Holland) as above and 50 ng/mL of commercial or synthetic VEGF-A were added. The flasks were incubated with anti-CD31 and anti-CD144 fluorescent antibodies as specific EC markers in the dark for one hr. Untreated cells were used as negative controls. Fluorescent images were captured on a Nikon Eclipse TS100 microscope.

Statistical analysis

The experiments were performed in duplicate. The statistical analysis included the independent sample t-test to evaluate differences between variables in each group. The studied groups were

compared using the Mann–Whitney U-test. All data were expressed as means ± SDs using SPSS statistic 16.0 software program (IBM Corporation, NY, USA). P values < 0.05 were considered statistically significant.

Results

### Evaluation of expressed protein in different hosts

The SDS–PAGE showed a more intense band representing recombinant VEGF-A in *E. coli* BL21 (DE3) (0.8 mg/mL) than in pLysS (0.1 mg/ml) (Fig. 1).
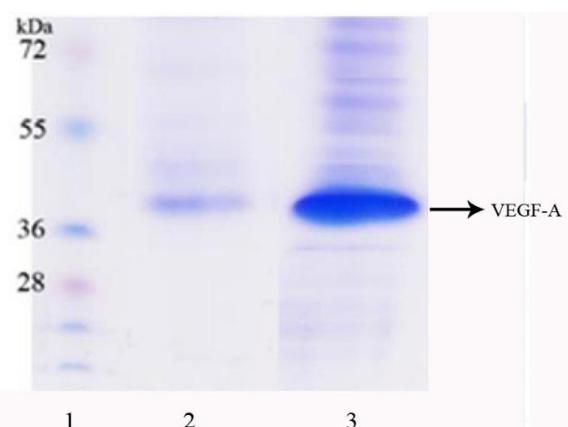
Fig. 1. 40 kDa purified proteins in pLysS and DE3 (Lane 1: Marker, Line 2: pLysS, Line 3:DE3)

Purified protein

After dialysis of the expressed-protein in *E. coli* BL21 (DE3) using PBS buffers of different pHs, the most intense 40 kDa band was seen on SDS-PAGE from protein dialyzed at pH 8 for 24 hours at 4 °C with shaking (Fig. 2A). The anti-VEGF antibody bound to a similar-sized protein on western blots (Fig. 2B).
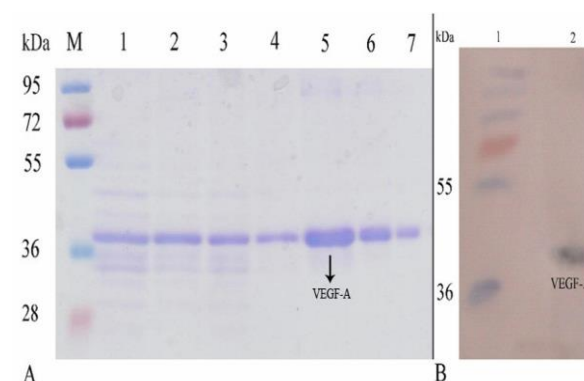

Fig. 2. Purified proteins in *E. coli* BL21 (DE3). (A) SDS-PAGE of dialyzed protein, M; size marker, lane 1; pH 6, lane 2; pH 6.5, lane3; pH 7, lane 4; pH 7.5, lane 5; pH 8, lane 6; pH 8.5, lane 7; pH 9. (B) Western blot, lane 1; size marker, lane 2; synthetic VEGF-A-antibody reaction.

Effect of the recombinant protein on MSC differentiation into ECs

The EC-specific markers CD31 and CD144 were more highly expressed on MSCs incubated with synthetic VEGF-A than on those incubated with commercial VEGF-A ($p < 0.05$). The specific MSC markers CD90, CD73, and CD44 were more highly expressed in MSCs incubated with commercial VEGF-A than those incubated with synthetic VEGF-A ($p < 0.05$). Furthermore, CD34 was expressed at a lower level in MSCs incubated with synthetic VEGF-A than those incubated with the commercial protein. CD105 expression was not significantly different between MSCs incubated with the different VEGF-As (Fig. 3). Fluorescence microscopy imaging showed greater CD 31 expression in cells incubated with synthetic VEGF-A than in those incubated with commercial VEGF-A (Fig. 4).
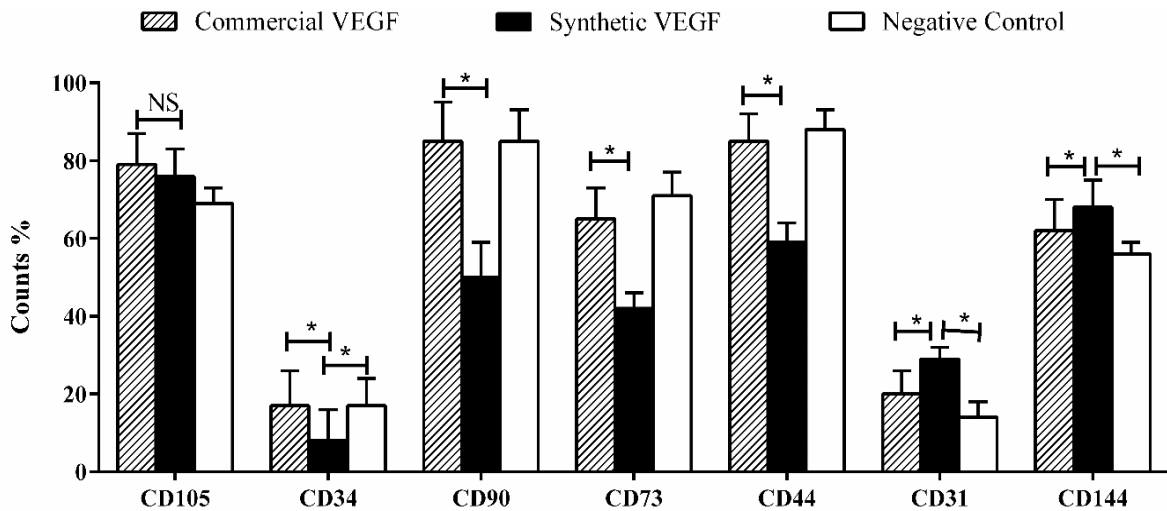
Fig. 3. Counts % of CD markers in MSC cultures at 10-day differentiation. *; $p < 0.05$, NS; Not significant.
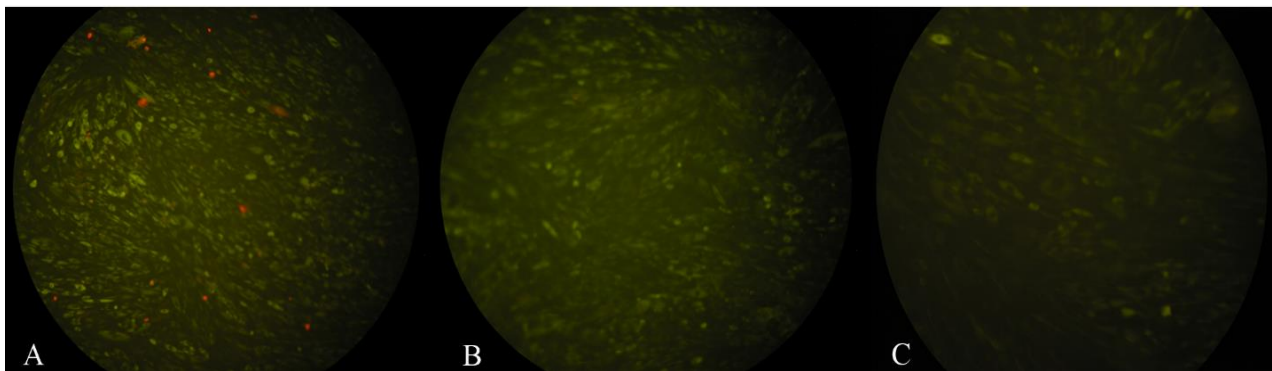
Fig. 4. MSCs differentiate to ECs and express CD31 10 days after VEGF-A treatment. Images were captured by fluorescent microscopy. (A) CD31 expression in MSCs after incubation with recombinant VEGF-A, (B) CD31 expression in MSCs after incubation with commercial VEGF-A, and (C) negative control. Magnification: ×10.

Discussion

VEGF-A, the most important member of the VEGF family, mediates angiogenesis and cell differentiation (1-4, 6-8, 17). In this study we produced recombinant VEGF-A in a bacterial expression system and investigated its ability to induce differentiation of MSCs to ECs.

Our study showed that recombinant VEGF-A was more effective than commercial VEGF-A in activating differentiation of MSCs to ECs. In addition, expression of the EC-specific markers CD31 and CD144 was greater in recombinant VEGF-A-treated MSCs than in MSCs treated with the commercial protein. On the other hand, the MSC markers CD90, CD73, and CD44, and the bone marrow stem cell marker CD34 were all expressed at significantly lower levels in recombinant VEGF-A-treated cells than those treated with the commercial protein, confirming

the effectiveness of synthetic VEGF-A in MSC differentiation.

In a previous related study, a high yield of glycosylated-recombinant VEGF-A was obtained from Chinese hamster ovary (CHO) cells using a bioreactor (18). The different VEGF-A concentrations in their study and ours (80 vs. 0.8 mg/L) may be due to the different expression systems used and posttranslational modifications of the product.

In the Lee et al. study, MSCs were obtained from femur bone marrow of Yucatan microswine that were treated in vitro with commercial VEGF-A along with interleukin (IL) 6, tumor necrosis factor (TNF) -α and angiotensin (Ang) II. This study showed that IL-6 and TNF-α, in the presence of recombinant VEGF-A, inhibit MSC differentiation into ECs; however, Ang II promotes MSC differentiation into ECs. Our flow cytometry results agreed with those of Ikhapoh et al.; although differences between the studies included their use of cytokines along with commercial VEGF-A (2).

In the Ai et al. study, the roles of Smad2/3 and Smad1/5/8 signaling were evaluated in MSCs differentiation to ECs. The Smad2/3 pathway was activated by TGF-β1 and Smad1/5/8 by bone morphogenetic proteins (BMPs). They concluded that the Smad2/3-activated group was 10% positive for CD31 cells. BMP9 activation in low Smad1/5/8 phosphorylation doubled the percentage of CD31-positive cells but high phosphorylation and BMP9 were ineffective. In contrast, Smad2/3 acted first as a blocker and then as an activator in MSC differentiation. Conclusively, low Smad1/5/8 phosphorylation was more effective than R-Smad in inducing VEGF-A expression and MSCs differentiation into ECs (19).

Ikhapoh et al. reported that Rho/ROCK (members of a small GTPases family) signaling has an important role in VEGF-A expression and induction of endothelial cell migration and angiogenesis. Rho and ROCK inhibitors blocked the signaling mechanism responsible for differentiation of MSCs into ECs (20). The authors concluded that Rho/ROCK signaling controls myocardin-related transcription factor-A (MRTF-A), which act as an effector in VEGF-A-controlled differentiation of MSCs into endothelial cells.

Fernandez-Alonso et al. reported that regulation of VEGF-A and TGF-β signaling by p73 is necessary for EC differentiation, migration, and formation of vascular networks (7). Their data demonstrated that p73 in differential regulation is necessary for natural vasculogenesis and angiogenesis and p73 overexpression enhances tumor progression.

In another study, no paracrine signaling function was found for VEGF-A in cell surface receptor activation. Instead, the mechanism appears to utilize intracellular VEGF-A in controlled differentiation of osteoblasts and adipocytes by Lamin A and VEGF-A through regulating the levels of Runx2 and PPARγ transcription factors, respectively (1). Based on this information, it was speculated that bone marrow mass may be repaired by effects of intracellular VEGF-A in MSCs of bone marrow during aging.

Izuagie et al. evaluated the role of synergistic effects of VEGF-A and Ang II in MSCs differentiation to ECs. They found that the increase of AngII in relation to the potency of type I receptor (AT1R) is more important than AT2R (2, 20). In another study, intervention with TGF-β and VEGF-A identified RhoA/Poch as the molecular switch for MSC differentiation. If the molecular switch is active, MSCs will differentiate to fibroblasts but not to ECs. Furthermore, RhoA activation will induce matrix metalloproteinase-3 (MMP3) and consequent VEGF-A release, and MSC to EC differentiation (21, 22).

Kai Jin et al. reported that VEGF-A-induced MSCs differentiation to ECs increased vascularization. In this study, researchers fabricated a collagen-heparin multi-layer system as MSCs differentiate to ECs in mesh. Western blotting, immunofluorescence staining, and quantitative real-time polymerase chain reaction (qRT-PCR) were applied to evaluate the MSC differentiation rate and level of vascularization. They showed that expression of the endothelial differentiation markers CD31, Flk-1, and von Willebrand factor (vWF) were enhanced in a dose dependent manner in the collagen-

heparin/VEGF/MSCs system. MSCs differentiation into ECs was evaluated both in vivo and in vitro on the mesh (23). With respect to this study, our recombinant VEGF-A expressed in a recombinant by expression prokaryotic system can be used to study MSCs differentiation to ECs in research and likely therapeutic applications.

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