

Overproduction of Recombinant Human VEGF (Vascular Endothelial Growth Factor) in Chinese Hamster Ovary Cells

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Vascular endothelial growth factors (VEGFs) are a family of proteins that mediate angiogenesis. VEGF₁₆₅ is a VEGF-A isoform and has been extensively studied owing to its potential use in therapeutic angiogenesis. This study established Chinese hamster ovary (CHO) cells overexpressing recombinant human VEGF₁₆₅ (rhVEGF₁₆₅) protein. The production rate of the established CHO cells was over 80 mg/l of rhVEGF₁₆₅ protein from a 7-day batch culture process using a 7.5-l bioreactor with a 5-l working volume and serum-free medium. The rhVEGF₁₆₅ protein was purified to homogeneity from the culture supernatant using a two-step chromatographic procedure that resulted in a 48% recovery rate. The purified rhVEGF₁₆₅ protein was a glycosylated homodimeric protein with a higher molecular weight (MW) than the protein expressed from insect cells, suggesting that the glycosylation of the rhVEGF₁₆₅ protein in CHO cells differed from that in insect cells. The purified rhVEGF₁₆₅ protein in this study was functionally active with a half-maximal effective concentration of 3.8 ng/ml and specific activity of 2.5×10^5 U/mg.

Keywords: VEGF, Chinese hamster ovary (CHO) cell, recombinant protein expression, stable cell line, bioreactor process

Angiogenesis is the formation of capillaries or new blood vessels from preexisting vessels [24], and recognized as an important process in pathological tumor growth and metastasis, neovascular age-related macular degeneration, and rheumatoid arthritis [4, 10]. The primary mediator of angiogenesis is a family of proteins known as vascular endothelial growth factor (VEGF) [26], and seven glycoproteins have already been identified in this family of proteins:

VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and the placental growth factor (PlGF) [27]. Among these proteins, VEGF-A has been best characterized as it is considered to be most strongly associated with angiogenesis, and thus has been the target of anti-VEGF treatments [2, 7] and therapeutic angiogenesis [11]. Six VEGF-A isoforms (VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₃, VEGF₁₈₉, and VEGF₂₀₆) produced by alternative splicing have already been reported [8]. Yet, since VEGF₁₆₅ is known to be the predominant VEGF-A isoform in the human body, most therapeutic angiogenesis for treatment of tissue ischemia has been focused on the transfer of the VEGF₁₆₅ gene [11, 14] and treatment of the recombinant VEGF₁₆₅ protein [13].

Several attempts have been made to express recombinant human VEGF₁₆₅ (rhVEGF₁₆₅) as an active protein using *Escherichia coli*, yeast, and insect cell expression systems [19, 20, 25]. VEGF₁₆₅ is a homodimeric glycoprotein [9]. Unglycosylated rhVEGF₁₆₅ produced from *E. coli* was previously reported to be functionally equivalent to the native form in biological activities [28] and even entered clinical development [13], but it still differed from the natural forms of VEGF₁₆₅ present in the human body. Furthermore, production of rhVEGF₁₆₅ in *E. coli* requires a refolding process, as it is expressed in an inclusion body [25]. Yeast and insect cells are not suitable hosts for expressing human glycoproteins, as the glycosylation in these host cells is remarkably different from that in humans [12]. Although the glycosylation of VEGF₁₆₅ is not necessarily critical for its functional activities [28], using a recombinant protein with a structure equivalent to the native form is still desirable for therapeutic utilizations. Attempts have been made to express rhVEGF₁₆₅ in mammalian cells using adeno-associated viral vectors; however, the expression levels obtained were not industrially applicable [3]. Within the last ten years, Chinese hamster ovary (CHO) cells have become the most widely used host system for recombinant protein production, not only because CHO cells facilitate

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adequate posttranslational protein modifications, but also because they can be used in industrially applicable manufacturing processes. Accordingly, this study established a Chinese hamster ovary (CHO) cell line that overexpressed biologically active rhVEGF₁₆₅, along with industrially applicable bioreactor and purification processes.

The rhVEGF₁₆₅ expression plasmid, pMSG+VEGF(165), was constructed by introducing cDNA encoding human VEGF₁₆₅ under the SV40 promoter of pMSG, which was recently developed as an expression vector containing the human β -globin matrix attachment region [17]. The cDNA encoding human VEGF₁₆₅ was obtained from the Bank for Cytokine Research (Chonbuk National University, Korea) and the protein-coding region of this cDNA reamplified by PCR using the following oligonucleotide primers: 5'-CTAGCTAGCCACCATGAACCTTCTGCTGTCTTGGG-3' (sense primer: NheI recognition site underlined) and 5'-GAAGATCTCACCGCCTCGGCTTGTAC-3' (BglII recognition site underlined). The Kozak sequence (GCCACC) was added to the sense primer to create an efficient start for the translation [18]. The amplified DNA fragment was digested with NheI and BglII, and then ligated to the vector digested with the same enzymes. The resulting expression plasmid construct, pMSG+VEGF(165), was completely sequenced to confirm that it contained the entire human VEGF₁₆₅ coding region.

Dihydrofolate reductase (DHFR)-deficient CHO cells (DG44) were used as the host for expression of rhVEGF₁₆₅. The cells were cotransfected with 2 μ g DNA that consisted of a 100:1 molar ratio of the expression plasmid, pMSG+VEGF(165), and the selection marker plasmid, pDCH1P [5], using DOSPER (Boehringer Mannheim), as previously described [16, 22]. Thereafter, the transfected cells were cultured in an MEM- α medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) (Hyclone). Forty-eight hours after transfection, the cells were cultivated in a selection medium, MEM- α medium without any nucleoside (Gibco BRL) and supplemented with 10% heat-inactivated dialyzed FBS (Hyclone), for approximately two weeks, and then the stable transfectants were maintained for two weeks in the presence of MTX (Sigma-Aldrich Co.) at a concentration that increased in a stepwise fashion (10 nM \rightarrow 100 nM \rightarrow 1 μ M \rightarrow 5 μ M). The expression of rhVEGF₁₆₅ was measured by ELISA using DuoSet for hVEGF (R&D Systems). Since an additional increase of MTX to 10 μ M did not increase expression of rhVEGF, single-cell-derived clones were isolated from the stable transfectants adapted to 5 μ M MTX [1, 21]. The final rhVEGF₁₆₅ production rate obtained from the most productive cell line was \sim 25 μ g/10⁶ cells/day.

To adapt the cell line producing rhVEGF to a serum-free suspension culture, the most productive cell line was inoculated into 100 ml of protein-free medium, HyQ SFM4CHO (Hyclone), supplemented with 0.15% sodium

bicarbonate at a seeding density of 5×10^5 cells/ml in a 250-ml spinner flask (Bellico Biotechnology). When the cells reached a density of 1.0 – 2.0×10^6 cells/ml, they were subcultured at a seeding density of 5.0×10^5 cells/ml in 100 ml of a fresh protein-free medium with 0.15% sodium bicarbonate. The cells were then repeatedly subcultured during the mid-logarithmic growth phase for 30 days, thereby adapting the cells to a serum-free suspension culture. After harvesting these cells, they were resuspended in a serum-free Cell Freezing Medium (Sigma), and frozen in a freezer unit (Nalgene Cryo 1°C Freezing Container) for future use.

To measure the productivity of the selected rhVEGF₁₆₅-expressing CHO cell line selected and adapted to serum-free suspension culture, batch cultures were performed in a bioreactor. The cells were inoculated into 5 l of protein-free medium, HyQ SFM4CHO (Hyclone), supplemented with 0.15% sodium bicarbonate at a seeding density of 5.0×10^5 cells/ml in a 7.5-l bioreactor (New Brunswick Scientific). Fig. 1 shows cell growth and production profiles of rhVEGF₁₆₅ during a 7-day batch culture process in the 7.5-l bioreactor (working volume=5 l). The viable cell density reached at the highest level ($>3 \times 10^6$ /ml) on day 5, after which it began to decrease (Fig. 1). On the 7th day, cell viability decreased to 80%, at which point the bioreactor culture process was stopped. The amount of rhVEGF₁₆₅ accumulated in the medium during the 7-day batch culture process was up to 80 mg/l (Fig. 1).

A two-step chromatographic purification procedure was employed to purify the rhVEGF₁₆₅ protein from the culture

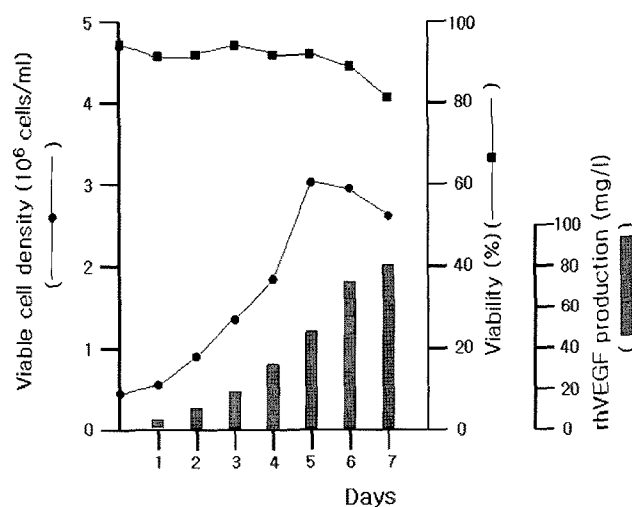


Fig. 1. rhVEGF₁₆₅ production profile of rhVEGF₁₆₅-expressing stable CHO cell line cultured in a 7.5-l bioreactor (5-l working volume) with serum-free medium.

The viable cell densities (-●-), cell viabilities (-■-), and accumulated rhVEGF₁₆₅ protein amounts in the culture medium (shaded rectangle) during the batch culture process are indicated. The amount of rhVEGF₁₆₅ accumulated in the medium during the 7-day batch culture process was up to 80 mg/l.

medium obtained from the bioreactor culture process according to procedures described previously [15, 23], with some modifications. Three hundred ml of the conditioned medium was diluted with distilled water (1:1) and loaded onto a 5-ml HiTrap-SP column (Amersham Biosciences) equilibrated with 10 mM sodium acetate buffer (pH 4.5). The bound proteins were then eluted with a step gradient of 0.4 M, 0.6 M, and 0.8 M NaCl in the 10 mM sodium acetate buffer (pH 4.5). The fractions containing the rhVEGF₁₆₅ protein were collected, the buffer changed to a PBS buffer, followed by loading onto a 1-ml HiTrap Heparin HP column (Amersham Biosciences) equilibrated with the PBS buffer. The bound protein was then eluted using a linear gradient of 0–1 M NaCl in the PBS buffer. Thereafter, the purified rhVEGF₁₆₅ protein was identified by SDS-PAGE under reducing conditions and shown to be composed of two protein bands of approximately 23 kDa and 18 kDa (Fig. 2A). The protein purity was greater than 96% and the recovery rate from the purification procedure about 48%. To investigate the nature of the two protein bands shown in Fig. 2A, the purified rhVEGF₁₆₅ protein was treated with peptide N-glycosidase F (PNGase F) and only one protein band detected (lane 5 in Fig. 2B), suggesting that the 23 kDa and 18 kDa protein bands correspond to glycosylated and unglycosylated forms, respectively (lanes 4 and 5 in Fig. 2B). The amount of the unglycosylated form was less than 10% of the glycosylated form (Fig. 2A).

The rhVEGF₁₆₅ protein purified in the present study was compared with those previously expressed and purified in *E. coli* (NIBSC) and insect cells (R&D Systems). When an SDS-PAGE analysis was conducted under reducing conditions, it was found that the *E. coli*-derived rhVEGF₁₆₅ protein only showed one protein band (unglycosylated) (lane 1 in Fig. 2B), whereas the insect cell-derived rhVEGF₁₆₅ protein showed two protein bands of approximately 20 kDa and 18 kDa (lane 2 in Fig. 2B). However, the two protein bands for the insect cell-derived rhVEGF₁₆₅ protein became one protein band of 18 kDa after treatment with PNGase F (lane 3 in Fig. 2B), suggesting that the 20 kDa and 18 kDa protein bands correspond to glycosylated and unglycosylated forms, respectively, as previously reported [19]. Yet, since the protein band patterns of the insect cell-derived rhVEGF₁₆₅ protein differed from those for the CHO-derived rhVEGF₁₆₅ protein (lanes 2 and 4 in Fig. 2B), this clearly indicates a different glycosylation of the rhVEGF₁₆₅ protein in the insect cells from that in the CHO cells. It is also possible that the glycosylation of the rhVEGF₁₆₅ protein in the insect cells was not as complete as that in CHO cells. The SDS-PAGE analysis under nonreducing conditions showed that the CHO-derived rhVEGF₁₆₅ protein formed a perfect homodimeric protein that was supposedly the active form (lane 3 in Fig. 2C). In addition, this homodimeric form of the CHO-derived protein has a higher MW than the *E. coli*-derived and insect

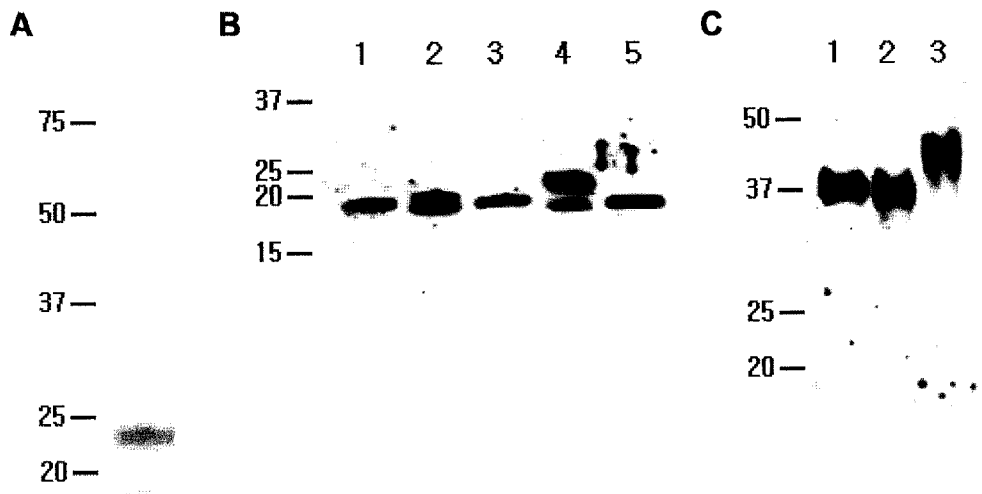


Fig. 2. SDS-PAGE analysis of purified rhVEGF₁₆₅ protein.

A. SDS-PAGE analysis of the purified rhVEGF₁₆₅ protein. Twenty ng of the purified rhVEGF₁₆₅ protein sample was separated by 10% SDS-PAGE under reducing conditions and visualized by silver staining. **B.** Comparison of purified rhVEGF₁₆₅ protein with *E. coli*-derived and insect cell-derived rhVEGF₁₆₅ proteins. The protein samples were separated by 10% SDS-PAGE under reducing conditions and analyzed by a Western blot analysis: lane 1, 10 ng of *E. coli*-derived rhVEGF₁₆₅ protein (NIBSC); lane 2, 50 ng of insect cell-derived rhVEGF₁₆₅ protein (R&D Systems); lane 3, 30 ng of insect cell-derived rhVEGF₁₆₅ protein (R&D Systems) treated with PNGase F; lane 4, 80 ng of CHO cell-derived rhVEGF₁₆₅ protein purified in this study; lane 5, 40 ng of CHO cell-derived rhVEGF₁₆₅ protein treated with PNGase F. The *E. coli*-derived and insect cell-derived rhVEGF₁₆₅ proteins contained bovine serum albumin as a stabilizer, and thus the proteins were visualized by a Western blot analysis using rabbit anti-VEGF antibody (Santa Cruz), peroxidase-conjugated anti-rabbit antibody (Polysciences Inc.), and SuperSignal West Pico Chemiluminescent Substrate (Pierce). **C.** Comparison of homodimeric forms of the purified rhVEGF₁₆₅ protein with those of *E. coli*-derived and insect cell-derived rhVEGF₁₆₅ proteins. The protein samples were separated by 10% SDS-PAGE under nonreducing conditions and analyzed by a Western blot: lane 1, 200 ng of *E. coli*-derived rhVEGF₁₆₅ protein (NIBSC); lane 2, 400 ng of insect cell-derived rhVEGF₁₆₅ protein (R&D Systems); lane 3, 400 ng of CHO cell-derived rhVEGF₁₆₅ protein purified in this study.

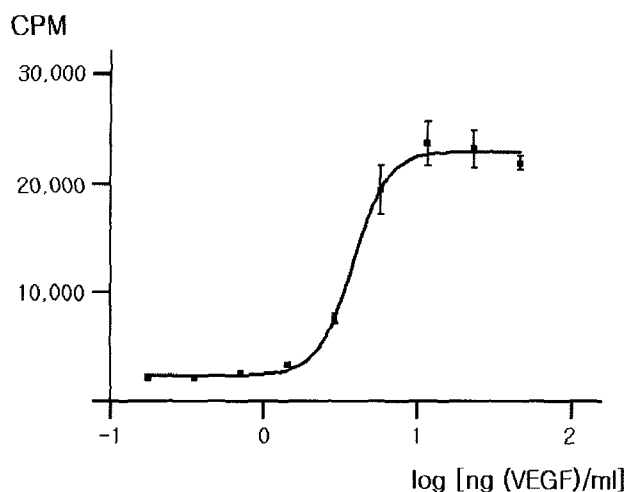


Fig. 3. Bioactivity assay of purified rhVEGF₁₆₅ protein. The biological activities of the purified rhVEGF₁₆₅ protein were measured by cell proliferation assay using HUVECs (see text). The error bar indicates the standard deviation. The half-maximal effective concentration was 3.8 ng/ml and the specific activity calculated as 2.5×10^5 U/mg.

cell-derived proteins, also confirming that the glycosylation of the rhVEGF₁₆₅ protein was different in the insect cells and CHO cells (Fig. 2C).

To evaluate the biological activity of the rhVEGF₁₆₅ protein expressed and purified in this study, a cell proliferation assay was performed based on a previously described method [6]. Human umbilical vein-derived endothelial cells (HUVECs) were seeded on 96-well culture plates at a density of 5,000 cells/well with various concentrations of purified rhVEGF₁₆₅ protein and cultured for 48 h at 37°C. A total of 0.5 μ Ci of ³H-thymidine was then added to each well, and its incorporation measured after 24 h of incubation. As shown in Fig. 3, the purified rhVEGF₁₆₅ protein stimulated the proliferation of HUVECs in a dose-dependent manner. The half-maximal effective concentration was 3.8 ng/ml and the specific activity calculated was 2.5×10^5 U/mg, proving that the CHO-derived rhVEGF₁₆₅ protein obtained in this study was functionally active with a similar biological activity to previously reported rhVEGF₁₆₅ proteins [19, 20, 25].

In conclusion, a CHO cell line overexpressing biologically active rhVEGF₁₆₅ protein was successfully established. In addition, the rhVEGF₁₆₅ protein purified from the 5-l bioreactor batch culture process was functionally active. Although the biological activities measured by a cell proliferation assay were all similar, regardless of the expression system, the CHO-derived rhVEGF₁₆₅ protein appeared to be the most similar recombinant protein to the native form, particularly in terms of glycosylation. Moreover, the unglycosylated *E. coli*-derived or differently glycosylated insect cell-derived rhVEGF₁₆₅ proteins could result in unexpected problems, such as immunogenicity, when treating human diseases. Although fine tuning is still required for industrial application,

the bioreactor culture and purification processes developed in this study would seem to be the best for the production of the rhVEGF₁₆₅ protein for therapeutic purposes.

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