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Production of functional human vascular endothelial growth factor$_{165}$ in transgenic rice cell suspension cultures

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ABSTRACT

Vascular endothelial growth factors (VEGFs) are secreted by tumor cells and other cells exposed to hypoxia, and play a critical role in the development and differentiation of the vascular system. In this study, we investigated the production of functional recombinant human VEGF$_{165}$ (rhVEGF$_{165}$) in transgenic rice cell suspension culture. Complementary DNA was synthesized from human leukemia HL60 cells and cloned into expression vectors under the control of the rice α-amylase 3D (RAmy3D) promoter. The rice seed (*Oryza sativa* L. cv. Dongjin) was transformed with this recombinant vector by the *Agrobacterium* mediated method and the integration of the target gene into the plant genome was confirmed by genomic PCR. The expression of rhVEGF$_{165}$ in the rice cells was determined by Northern blot and Western blot analyses. The accumulated rhVEGF$_{165}$ protein in the culture medium was 19 mg/L after 18 days of culturing in a sugar-free medium. The rhVEGF$_{165}$ was purified using a heparin HP column and its biological activity was tested on human umbilical vein endothelial cells (HUVECs). The purified rhVEGF$_{165}$ significantly increased the proliferative activity of the HUVECs. Therefore, it was demonstrated that functional rhVEGF$_{165}$ could be produced using transgenic rice suspension culture vector under the control of the RAmy3D promoter.

1. Introduction

During the last 20 years, above the limitations of low expression levels, whole plants and plant cells have been considered as competitive expression systems for the production of numerous human recombinant proteins of therapeutic and pharmaceutical value, including cytokines, enzymes, hormones, growth factors, and antibodies [1,2]. Plant-based systems have become promising systems for the production of heterologous proteins; their advantages include the ease and low cost of scaling up biomass production, the capacity for the accurate post-translational modification of eukaryotic proteins, and the low risk of contamination by animal pathogens [2–4]. In the trend of the utilization of plant cells for the production of foreign proteins, a rice cell suspension culture system has been developed as an alternative system for the production of a number of biologically active human proteins and antibodies such as α$_1$-antitrypsin [5], lysozyme [6], interferon-gamma [7], colony stimulating factors [8,9], serum albumin [10], and interleukin-12 [11].

The use of the promoter, signal sequence, and terminator of amylase 3D gene from rice gives the rice cell culture system a huge advantage in reducing the total production cost in comparison to a whole plant platform, especially at the purification stage [2]. Among members of the amylase multigene family, amylase 3D gene expresses an alpha isozyme from the scutellum of rice seed (named as RAmy3D) during germination. The RAmy3D gene is also expressed in the suspension cells derived from the scutellum in response to sugar deprivation. This essential point has been applied in recombinant protein production by growing the transformed rice cells efficiently to a high density in media containing a high sucrose concentration before switching the transgenic rice cells to sucrose-free media [12,13]. Under the control of the RAmy3D promoter and 3D signal peptide, recombinant proteins can be induced

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and secreted into the surrounding media under the sucrose-free conditions. This feature simplified the purification process and minimized contamination with other proteins from the host.

Vascular endothelial growth factors (VEGFs) are a family of proteins that mediate angiogenesis. A well-documented in vitro activity of VEGF is the ability to promote the growth of vascular endothelial cells derived from arteries, veins, and lymphatics. A single human VEGF gene, by alternative mRNA splicing, can give rise to at least six protein isoforms, including $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$. $VEGF_{165}$, the most abundant and best characterized isoform, shows strong mitogenic potency toward vascular endothelial cells, both under physiological and pathological conditions [14–17]. Recent studies have demonstrated that $VEGF_{165}$ could be produced in bacteria [18], yeast [19], Chinese hamster ovary cells [20], insect cells [15], and silkworm [21] culture systems.

In this study, we report that $VEGF_{165}$ could be produced using a transgenic rice suspension culture under the control of the RAmy3D promoter and that the activity of $VEGF_{165}$, which was purified by a heparin HP column, was confirmed by its proliferative activity on HUVECs.

2. Materials and methods

2.1. Isolation of human $VEGF_{165}$ cDNA

Total RNA was isolated from human leukemia HL60 cells as described previously [22] and used as a template in a reverse transcription (RT) polymerase chain reaction (PCR) to amplify the $VEGF_{165}$ cDNA gene without its signal sequence. A pair of primers for the PCR amplification were designed according to the *Homo sapiens* VEGF isoform $VEGF_{165}$ mRNA (GenBank accession no. AF486837.1), which included a forward primer (5′-GCACCCATGGCAGAAGGA-3′) and a reverse primer containing the *Kpn*I restriction site (underlined) (5′-GGTACC CTA CCGCCTCGGCTTGTCACA-3′). PCR was performed for 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s in a reaction mixture containing 5 μL of the above cDNA solution, 5 units of *Taq* DNA polymerase, 12.5 mM dNTP, and 10 pmol of each of the primer sequences. The resulting 420-bp PCR fragment contained the open reading frame for $hVEGF_{165}$ lacking its signal peptide. To fused with rice amylase 3D signal sequence, rice amylase 3D signal sequence was amplified with forward primer containing the *Bam*HI restriction site (underlined) (5′-GGA TCC ATG AAG AAC ACC AGC AG-3′) and a reverse primer (5′-GGC TTG GCC CGA GTT CAAGGT C-3′) [8]. The $hVEGF_{165}$ fused with the signal sequence of rice amylase 3D (Ramy3D) was introduced into pGEM-T easy vector (Promega, Madison, MI) to generate the plasmid pMYD162. Its DNA sequence was verified by sequence analysis.

2.2. Construction of plant expression vector

In order to construct the plant expression plasmid pMYD171, the $VEGF_{165}$ fused with rice amylase 3D signal sequence in pMYD162 was digested with *Bam*HI and *Kpn*I and introduced into the same sites of the plant expression vector, pCAMBIA1300 [23], under the control of the RAmy3D promoter, with 3′UTR of the RAmy3D gene as the terminator. The hygromycin phosphotransferase (HPT) gene was used as the selection marker for hygromycin B (Fig. 1).

2.3. Rice transformation

The mature rice (*O. sativa* L. cv. Dongjin) seeds were prepared for callus induction as described by Chan et al. [24]. In brief, rice seeds were dehusked and washed several times with 70% ethanol, then washed with 50% NaOCl (Sigma), and this was followed by thorough rinsing with autoclaved distilled water and blotting on WhatMan filter paper. The sterilized seeds were germinated for three weeks on callus induction agar plates (N6 plates) containing 4 g/L of N6 medium salts (Duchefa, Haarlem, The Netherlands), 30 g/L of sucrose, 2 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D), 0.02 mg/L of kinetin, and 4 g/L phytagel. Approximately, 20 pieces of three weeks old embryogenic rice callus were isolated and cultured on a co-culture plate (N6 plate without hygromycin B), which was placed for 1 day in the dark before rice transformation mediated by *Agrobacterium*. The binary plant expression vector pMYD171 was transformed into *Agrobacterium tumefaciences* LBA4404 using the helper plasmid pRK2013 by the tri-parental mating method [25,26]. The newly generated calli were collected and placed on the plate containing 50 mg/L of hygromycin B antibiotic (A.G. Scientific Inc., San Diego, CA) for selection.

2.4. PCR of genomic DNA

To confirm the integration of the $VEGF_{165}$ gene into the rice genome, genomic DNA PCR was conducted. The putative transgenic calli, which were well grown on plates containing 50 mg/L hygromycin B, were collected for the extraction of genomic DNA by the method described by Aljanabi and Martinez [27]. The PCR was carried out with 20 μL of PCR mixture containing genomic DNA (200 ng), forward primer $VEGF_{165}$-F1 5′-GGATCCGCACCCATGGCAGAAGGA-3′, reverse primer $VEGF_{165}$-R1 5′-GGT ACC CTA CCG CCT CGG CTT GTC ACA-3′, and 10 μL of GoTaq® Green Master Mix (Promega U1100, Madison, WI, USA). The PCR products were then separated by electrophoresis on 1% agarose gel.

2.5. RNA isolation and Northern blot analysis

For Northern blot analysis, total RNA was isolated from suspension cells grown during 7 days in N6 (S-) liquid media using the TRI reagent (MRC Inc., Cincinnati, OH) according to the manufacturer's instructions. RNAs (20 μg) were size-fractionated by agarose gel electrophoresis and were then transferred to a nylon membrane (Amersham Pharmacia Biotech RPN82B, Piscataway, NJ, USA) using a capillary blotting system. This membrane was then hybridized with α^{32}P-labeled human $VEGF_{165}$ probe using the Prime-a Gene labeling system (Promega U1100, Madison, WI, USA) at a temperature of 65 °C in a Hybridization Incubator (FINEPCR Combi-H, Seoul, Korea). The membrane was washed twice in 2XSSC and 0.1% SDS, and then washed twice more with 2XSSC and 1% SDS for 15 min each at 65 °C. The hybridized bands were detected via autoradiography on X-ray film (Fuji Photo Film Co. HR-G30, Tokyo, Japan).

2.6. Establishment of rice cell suspension culture

Cell suspension cultures were generated from seven cell lines with high levels of mRNA. Transgenic calli were moved into 50 mL of N6 + S liquid medium containing 2 mg/L of 2,4-dichlorophenoxyacetic acid, 0.02 mg/L of kinetin, and 30 g/L of sucrose in a 300-mL flask. The suspension cultures were maintained on a rotary shaker (110 rpm) at 25 °C with dark condition and subcultured every 9 days by transferring cells into fresh medium. In order to investigate the $rhVEGF_{165}$ expression levels, five grams of fresh weight cell was cultured in 50 ml of N6 medium without sucrose for 7 days and culture medium was collected for further analysis.

2.7. SDS-PAGE and Western blot analysis

The expression of $VEGF_{165}$ protein was confirmed by SDS-PAGE and Western blot analysis [28]. The induced culture supernatant was collected and centrifuged at 15,000×*g* for 5 min to remove the rice cells. 5 μg of protein from the culture medium were separated via 12% (w/v) SDS-PAGE and were electroblotted onto a nitrocellulose membrane. The membrane was then incubated in blocking solution [5% (w/v) non-fat dried milk in TBST buffer (20 mM Tris–Cl, pH 7.5, 500 mM NaCl, and 0.05% Tween 20)], followed by goat anti-human $VEGF_{165}$ polyclonal antibody (BAF293, R&D Systems, Minneapolis, MN), followed by anti-goat IgG antibody conjugated with alkaline phosphatase (Sigma, St. Louis, MO, USA) as a secondary antibody. The commercial recombinant $hVEGF_{165}$ derived from insect cells (293-VE, R&D Systems) was used as positive controls. The gel was stained with 0.25% Coomassie brilliant blue R-250, with 45% methanol and 10% glacial acetic acid. Protein concentration was determined with the protein assay reagent (Sigma) based on the Bradford method, using bovine serum albumin (BSA) as a standard.

2.8. Quantification of $rhVEGF_{165}$ by enzyme-linked immunosorbent assay

The amount of $rhVEGF_{165}$ was measured by sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Primary monoclonal antibody against $rhVEGF_{165}$ (MAB293, R&D Systems) was used as the capture antibody at a concentration of 2 μg/mL in coating buffer. Fifty microliters of coating antibody were loaded into each well and the ELISA plate was incubated at 4 °C overnight. The capture antibody was flicked off and the plate was washed three times with phosphate buffered saline Tween-20 (PBST). To block non-specific binding sites, 200 μL of 1% BSA in phosphate buffered saline (PBS) were loaded into each well and held at room temperature (RT) for 2 h. The plate was then washed three times with PBST. Subsequently, 100 μL of diluted positive control $rhVEGF_{165}$ (293-VE, R&D Systems) and induced samples were loaded into each well and stood overnight at 4 °C. The plate was washed 3 times and 100 μL of goat-derived human $VEGF_{165}$ biotinylated affinity purified polyclonal antibody (BAF293, R&D Systems) at diluted concentrations of 0.1 μg/mL were added as detection antibody and incubated at RT for 1 h. The solutions in the wells were replaced with 100 μL of diluted 1:1000 avidin-horseradish peroxidase (554058, BD Pharmingen) in blocking solution and incubated at RT for 30 min. The plate was washed four times and 100 μL of a mixture of TMB substrate solutions (555214, BD Pharmingen) were added into each well and incubated at RT for 20 min for a color developmental reaction. This reaction was stopped by adding 50 μL of stopping solution (6.3% H_3PO_4). The absorbance of samples was recorded at 430 nm with a microplate reader.

2.9. Purification of $rhVEGF_{165}$

The $rhVEGF_{165}$ secreted in rice cell culture was purified using HiTrap Heparin HP column (GE Healthcare, Uppsala, Sweden) following the manufacturer's instructions. Sodium phosphate was added into the filtrated supernatant at a concentration of 10 mM (pH 7.0) before the supernatant was introduced into the heparin HP purification column. The bound proteins were eluted using a sodium phosphate binding