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Potential Application of Plant-derived Bioengineered Human VEGF for Tissue Regeneration

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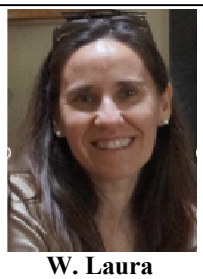

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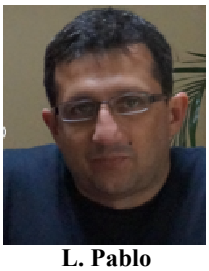

L. Pablo

Abstract: The design and development of novel biological drugs are among the most exciting new areas of biotechnology which are gaining the attention of scientists. In the last few decades several fabrication processes have been proposed and developed for the production of recombinant growth factors. However, traditional production processes have several limitations in terms of scale- up, cost-efficiency and purity grade of the proteins. In the present study, we propose for the first time the proof-of-concept of large-scale production of growth factors in plants as a new alternative to other production processes. We have decided to select vascular endothelial growth factor (VEGF) as model assuming its key role in cell survival and regenerative medicine. Results show that the present protocol is efficient to scale up a purification procedure of rh VEGF isoform 165 in *Nicotiana benthamiana* plants. Our procedure resulted in dimeric VEGF protein with high purity degree and yield, which showed full biological activity over endothelial and epithelial cells, suggesting great potential for its use in regenerative medicine. This protein could be exploited not only in tissue repair and regeneration but also as a biologically active ingredient in dermocosmetics.

Keywords: Growth Factor, recombinant human VEGF, plant expression, proteins, regeneration, cosmeceuticals.

INTRODUCTION

The impact of biotechnology on medicine is increasing progressively. Nowadays, it is believed that there are about a thousand of biotechnology-derived pharmaceuticals, including drugs and vaccines, under research or development processes. Moreover, it is estimated that these types of pharmaceuticals will reach a global market of 18 billion dollars in 2017.

The application of growth factors in different areas of regenerative medicine has acquired great relevance in recent years. More than 30 years have passed since the first FDA approval of the recombinant insulin, produced by bacterial expression systems in 1982. Different expression systems have been developed and improved over the years and today it is possible to manufacture a large number of biotechnological products of high purity and with excellent potential for therapeutic purposes. These products generated through biotechnological process, are named "biosimilars" and they must offer effectiveness and safety according to the regulatory requirements.

Although the bacteria-based production system has been one of the most frequently used to manufacture recombinant growth factors, it does not offer the potential to make all post-translational modifications necessary to produce biologically active proteins. Furthermore expression systems derived from mammalian cells, which are able to perform the posttranslational changes, are difficult to scale up and are significantly more expensive methodologies, leading to cost-efficiency limitations in the production of proteins and growth factors [1, 2].

Plants have emerged as a serious competitive force in the production of therapeutic and industrial proteins. They have a cellular machinery with the same biosynthetic pathways as any other eukaryotic expression system including mammalian and insect cells [3-5]. Plant-based expression systems are not contaminated by microbial toxins (endotoxins) or human pathogens (prions and virions), which are critical hurdles for therapeutic applications [6]. Moreover, transient expression, and particularly the use of plant virus-based expression vectors, offers several additional advantages. This method is based in an ancient biological systems, comprising the plants and their natural pathogens. A viral vector can be easily manipulated and the infection process in plants is a rapid

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mechanism that constitutes a valuable alternative for the production of recombinant proteins. In fact, plant viral vectors exploit on the one hand the effective viral replication and on other hand the plant cellular machinery, in this way they can produce a large quantities of recombinant proteins in a few weeks [7].

Several human proteins such as antimicrobials, cytokines, and antibodies have been produced using the plant virus-based expression vector [8]. The use of plants as protein biofactories is particularly relevant in the production of cytokines and growth factors. These are soluble proteins exerting a variety of cellular processes such as proliferation, migration and differentiation. All these processes play an essential role on tissues repair and regeneration.

Vascular Endothelial Growth Factor (VEGF) is one of the most studied growth factor in the literature. It is known to play a central role in the growth of blood vessels, lymphangiogenesis, vascular permeability and homeostasis of many organs [9, 10]. The VEGF family consists on five secreted proteins (VEGF-A, B, C, D and placental growth factor). In particular, VEGF-A is a homodimeric glycosylated protein of about 42-43 kDa with high homology to platelet-derived growth factor (PDGF) [11]. There are at least 13 isoforms of VEGF, produced by "alternative promoter usage, alternative splicing and alternative initiation" of the same gene [12] each one with structural differences which determine its function *in vivo*.

Recent evidence suggests that VEGF plays a critical role in regenerative medicine and tissue repair processes, not only by restoring the supply of oxygen when the blood circulation is inadequate, but also stimulating and increasing the survival of stem cells such as mesenchymal stem cells (MSCs) or stem cells derived from adipose tissue (ASCs). Some of these effects may be mediated by the stimulation of hydrogen sulfide synthesis from endothelial cells, which promote their proliferation and permeability. In addition, VEGF stimulates epithelialization and collagen deposition [13]. In fact, the increase of VEGF expression in wounds of mice is associated with an increment in epidermal and dermal proliferation, regeneration, angiogenesis, fibroblast proliferation and maturation of extracellular matrix [14].

In this study, we propose a proof-of-concept of a plant-based factory to produce and characterize pure and biofunctional VEGF. To address this, we have used an expression system derived from *Tobacco mosaic virus* (TMV) to provide large-scale production of biologically active VEGF (isoform 165). Furthermore, we have characterized the recombinant protein and we have evaluated whether plant-derived VEGF shows regenerative potential on epithelial and endothelial human cell lines. The results presented herein constitute the basis of a new technological platform for large-scale production of active VEGF with potential applications in regenerative medicine and/or dermo-cosmetic.

MATERIAL AND METHODS

Construction of the Expression Vector and Codon-harmonized Sequence of Human VEGF 165

The coding sequence of VEGF 165 isoform A (27-191aa –Swiss Prot Number P15692-4) was synthesized by GENEART AG optimizing the codon usage for *Nicotiana benthamiana* plants. The sequence has eight histidines at the N-terminus end. This DNA was inserted into TMV GENEWARE® vector under the control of a duplicated promoter of the virus CP gene, producing a sub-genomic RNA. TMV GENEWARE construction contains the coding sequence of the extensine signal peptide (PSN) from *Nicotiana tabacum*, which directs the recombinant human VEGF towards the endoplasmic reticulum, (Fig. **1**).

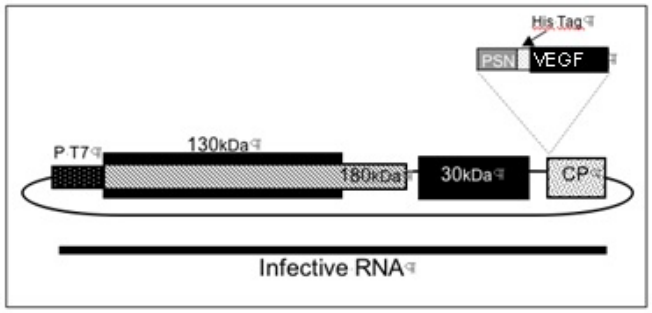

Fig. (1). Schematic diagram of the construction of the recombinant plasmid TMV-PSN-VEGF DNA clon. cDNA clones are transcribed from the T7 bacteriophage RNA promoter (PT7), followed by infection of Nicotiana plants. The genomic RNA is used as an mRNA and translated to produce the human recombinant VEGF. The codifying sequence of VEGF with a tag was inserted under the control of a duplicated promoter of the viral coat protein (CP) gene.

The optimized sequence has 173aa and the monomeric form has a molecular weight of 20.2 kDa, 7.65 as isoelectric point and its molecular formula is $C_{854}H_{1340}N_{274}O_{257}S_{22}$.

Plasmid DNA of the stable clone TMV-PSN-VEGF clone was purified using QIAGEN's plasmid purification kit, following the manufacturer's recommendations. For *in vitro* transcription (IVT) reactions we used AMBION's high yield capped RNA IVT mMESSAGE mMACHINE® T7 kit, according to Williams *et al.,* 2014.

Nicotiana Plants Inoculation with IVT RNA

Each young plant of 18-28 days was inoculated by gently scraping their leaves with 50 µL of diluted IVT RNA (2.5 µL of IVT reaction in 100 µl final volume of 0.043 M glycine, 0.139 M K_2HPO_4, 1% sodium pyrophosphate, 1% bentonite and 1% of celite). Plants were then incubated at 25ºC in chambers with 60% humidity and a photoperiod of 16/8 hours light/darkness. The infectivity of the recombinant virus and the stability of the insert were evaluated by RT-PCR according to Williams *et al.,* 2014, using oligonucleotides cTMVF 5'-CGT GTG ATT ACG GAC ACA ATC C-3' and cTMVR 5'-TAC TGT CGC CGA ATC GGA TTC G-3', which are flanking the VEGF insert.

These plants were pooled and used as inoculum to amplify the infection in a new set of plants from which recombinant human VEGF 165 was purified.

Protein Purification and Yield Evaluation

The first stage of purification was performed by affinity chromatography (IMAC- resin Ni2+ Sepharose Fast Flow, GE) according to Williams *et al*., 2014.

Starting material for protein purification was typically 300 g of infected leaves, grounded in a blender during 3 min in 1.2 L of buffer A: 10 mM potassium phosphate buffer pH 7.3-7.6, 100 mM NaCl, 0.05% triton x100 and 0.1 mM PMSF. The plant extract was filtered and incubated in batch with Ni2+ Sepaharose resin (GE Healthcare), 1h at room temperature with 15 mM imidazole. Elution was then performed with 100 mL of buffer A + 250 mM imidazole. The elution was dialyzed against 10 mM potassium phosphate buffer, 50 mM NaCl pH 6.8, and concentrated to 70 mL. The recombinant protein was purified by FPLC in an AKTA Explorer (GE) using cations exchanges columns (HiTrap SP sepharose FF). Aliquots of 1 mL were recovered. The peaks were analyzed on a 0.1% SDS, 12.5% PAGE [15] and either Coomassie stained or transferred into a nitrocellulose membrane [16] using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Inc.). The membrane was then blocked with 5% skim milk, 1.25% Glycine, 150 mM NaCl in sodium phosphate buffer pH 7.4, and probed with a monoclonal antibody anti His Tag (Clontech Inc.), diluted 1:5,000, followed by treatment with an alkaline-phosphatase-conjugated rabbit anti-mouse IgG (Zymed), diluted 1:200.

The selected bands from the Coomassie gel were cut off from the gel and tryptic fragments were analyzed by Matrix-Assisted Laser Desorption/Ionization Time Of Flight (MALDI-TOF).

Endothelial Cells Proliferation Activity Induced by Plant rh VEGF-165

The biological activity of the purified recombinant VEGF-165 was determined by MTT proliferation assay using human endothelial cells (HUVEC). Human umbilical vein cells were grown in F12K medium (LONZA) supplemented with 10% (v/v) FBS, 1% penicillin-streptomycin glutamine, 0.1 mg/mL heparin and 0.05 mg/mL endothelial cell growth factor supplement (ECGS). HUVEC were subcultured into 96-well plate at density of 4,000 cells per well in the growth medium overnight. Next, medium was replaced by F12K medium without ECGF and 0.2, 0.5, 1, 2, 4 and 8 ng/mL of plant recombinant VEGF. A cell proliferation assay (MTT) based on the reduction of tetrazolium salt to formazan by mitochondrial dehydrogenases in viable cells was used to determine number of cells at 48 hours. As a positive control, commercial VEGF from *E. coli* expression system was used. Proliferation of cells in medium without VEGF was set to 100% (basal activity).

In vitro Human Keratinocytes Wound Healing Assay

Human keratinocytes (HaCaT) were seeded in a 6-well plate and grown up to 90% confluence in DMEM 4.5 g/L glucose with 10% FBS medium. To mimic the injury, a cell scraper was used. The scratch was made like a line that completely crossed the well. After that, cellular debris was removed and the medium was changed to DMEM 4.5g/L glucose with a low dose of FBS (5%). Different concentrations of rh VEGF were added (5, 10 and 50ng/mL). As a positive control 5ng/mL of GM-CSF were used. This is a potent stimulator of cellular migration. Cells that were not exposed to VEGF were considered as the negative control. Pictures were taken immediately after growth factors addition (T0) and 24h after *in vitro* culture, (Fig. **5**).

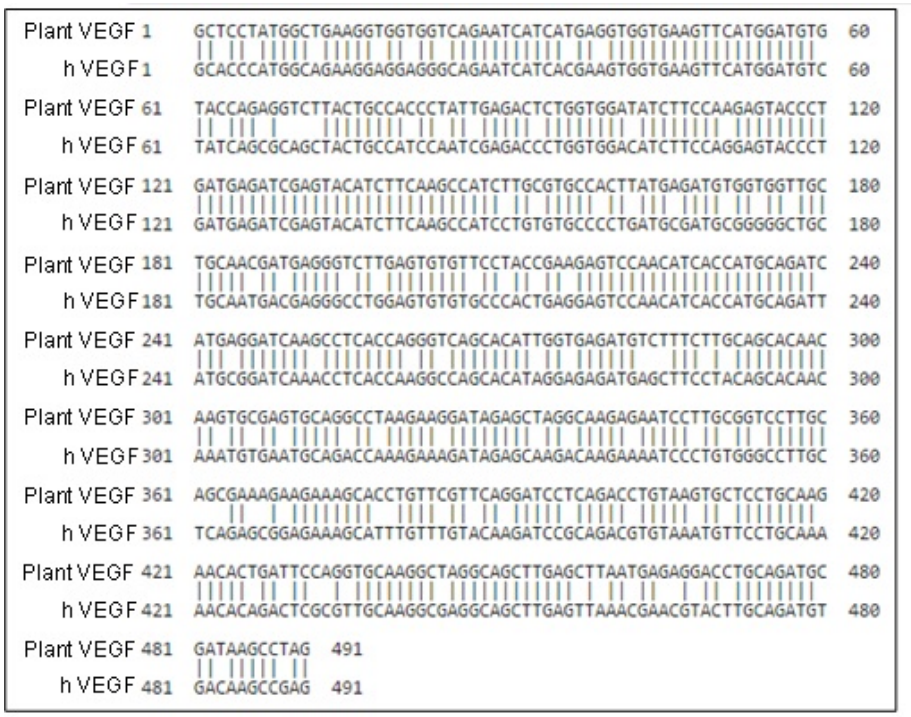

Fig. (2). Codon harmonization improves expression level of Human VEGF in Plants. Upper: Optimized coding sequence of rh VEGF (*plant VEGF*) expressed in *Nicotiana benthamiana* plants. Lower: human VEGF (*h VEGF*) sequence, accession number NP_001165097.

RESULTS

Construction of the Expression Vector with Codon-Harmonized Sequence

Recombinant human VEGF-165 optimized coding sequence was cloned in the TMV-PSN-VEGF expression vector and expressed in *Nicotiana benthamiana* plants. Figure **2** shows an alignment of optimized VEGF-165 coding sequence against human VEGF-165 sequence.

One hundred *Nicotiana benthamiana* plants were inoculated with the construction TMV-PSN-VEGF. Symptoms of infection were visible after 5-6 days on 100% of the plants as reflected by the mild leaf deformation with variable mottling grade. Two weeks after inoculation, the stability of the construction was analyzed by RT-PCR assay, including a negative control (TMV clone, empty vector) in the assay. Results observed on agarose gel reflected only one band of 754 bp that corresponds to the expression vector plus the insert sequence (rh VEGF, data not shown).

Process of Purification and Characterization of Plant-derived VEGF

We designed and set up a purification protocol that allows the recovery of 50 mg of active recombinant protein per kg of fresh plant tissue, increasing the purity over 97%.

On the first stage of purification an affinity chromatography (IMAC-resin Ni2 + Sepharose Fast Flow, GE) was performed according to Williams *et al*., 2014, in which a 50% of purity was achieved (60-70 mg of recombinant VEGF partially purified). The second purification stage consisted on a cation exchange chromatography (SP) in which different fractions were separated (Fig. **3**). Those fractions (from 11 to 18) were concentrated and analyzed by 15% SDS-PAGE (reducing and no reducing condition, Fig **4 A** and **B**).Under reducing conditions, four bands between 15 kDa to 22 kDa were observed (Fig. **4A**). All of them were identified as VEGF through serological test with specific IgG and MALDI-TOF assay showing the same peptide mass finger printing pattern (data not shown). Furthermore, these peptides were compared against protein sequence databases (SwissProt with GPS software) reasserting identification of each band as rh VEGF. In addition, as illustrated in (Fig. **4B**), the dimeric form of the molecule, 40-44 kDa was clearly observed.

Proliferation of Endothelial Cells Induced by Plant-derived VEGF

The biological activity of the purified rh VEGF-165 was determined by MTT proliferation method on HUVEC. VEGF stimulated HUVEC proliferation on a dose-dependent manner, exerting maximal proliferation at 48 hours after treatment compared to negative control (Fig. **5**). An effective dose (ED50) $\leq$ 2 ng/mL was observed. Both, plant and bacterial-produced VEGF have similar dose-dependent activity patterns, but at the same concentration (ng/mL), plant-VEGF achieved a higher cell proliferation level.

In vitro Wound Healing Assay in Human Keratinocytes

In a second set of experiments, the potential of plant-derived rh VEGF to promote cell proliferation in human keratinocytes (HaCaT) was evaluated. Migration of HaCaT cells was promoted in a dose-dependent manner after treatment with rh VEGF (Fig. **6**). In fact, plant-derived rh VEGF promoted HaCaT cell migration, closing completely 1 mm wounds in 24 hours. Moreover, the high purity and potential of rh VEGF guaranteed activity at very low concentrations (5 ng/mL). The negative control did not contain any growth factor whereas the positive control used 5 ng/mL of granulocyte macrophage colony stimulating factor (GM-CSF), which is also a potent stimulator of cellular migration. These results highlights the regenerative properties of VEGF, based on its promoting effect on skin cell migration, proliferation and survival.

DISCUSSION

Biotechnology is currently the vanguard of promising technological trends, especially concerning the bioscience and technology fields as a whole. Design and development of novel biological medicines is one of the exciting new areas of biotechnology, which is gaining the attention of scientists. These new biologically active substances have a different nature compared to those produced through traditional chemical synthesis, that allows them to carry out a more precise and effective approach in certain disorders. These biologically

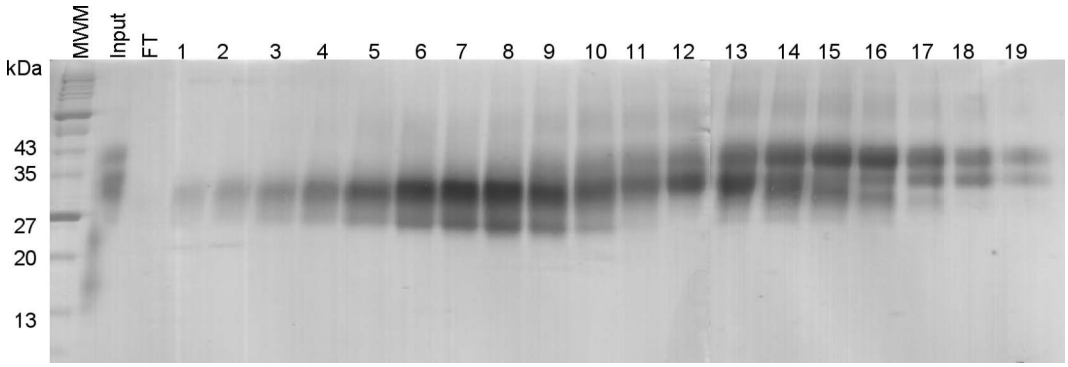

Fig. (3). Purification by Cation Exchange Chromatography. Elution from affinity purification was dialyzed and applied to a Hi Trap SP FF. VEGF was eluted with a linear gradient 0-1M of NaCl. 15% SDS-PAGE (non reducing conditions). MWM: Molecular weight marker (kDa), Input: Affinity purification input, FT: flow-thorugh of IEX-SP column, lines 1-19: different fractions obtained from NaCl gradient.

active substances known as "biosimilars" have already become the first treatment available for some age-derived diseases. Furthermore, biosimilars can be considered as key ingredients of new areas of medicine, such as regenerative medicine. Nowadays, thousands of drugs and vaccines of biological origin are in research and development phases, and it is estimated that in 2016 the biological drugs will become top-selless, reaching a global market of 18 billion dollars in 2017 (http://www.farmaindustria.es).

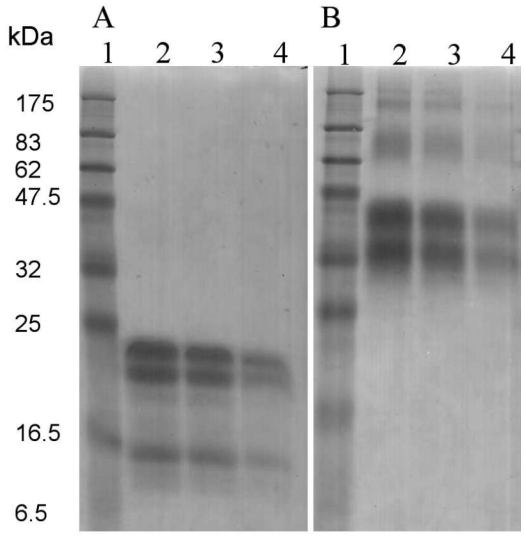

Fig. (4). Acrylamide electrophoresis in reducing and non-reducing conditions. Samples were loaded in 15% polyacrylamide gel and stained with coomassie blue. **A**: SDS-PAGE analysis of recombinant human VEGF-165. Lane 1 MWM: Molecular weight marker (kDa); Lane 2, 3, 4 contains 1, 0.6 and 0.3 ug of rh VEGF. **B**: The recombinant protein migrates with an apparent molecular mass of 35-40 kDa under no reducing conditions. Lane 1 MWM: Molecular weight marker (kDa); Lane 2, 3, 4 contains 1, 0.6 and 0.3 ug of rh VEGF.

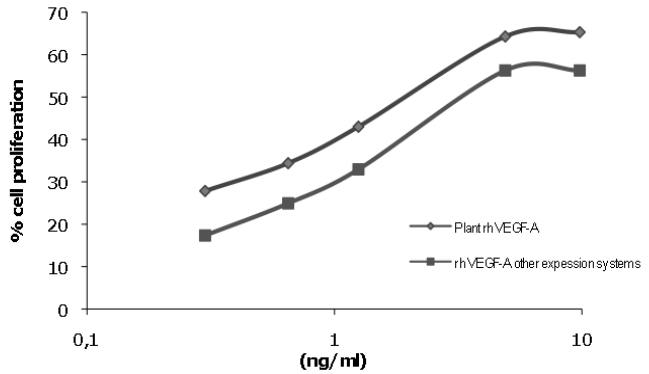

Fig. (5). Biological activity of plant-produced rh VEFG and prokaryotic-derived rh VEGF using a MTT assay on Human Umbilical Vein Endothelial Cells (HUVEC).

Growth factors are essential molecules in cell-to-cell communication and regulate many of the biological processes of tissue repair and regeneration. In the last few decades several methodologies have been proposed and developed for the production of recombinant growth factors. However, the traditional production processes have several limitations in terms of scale-up, cost-efficiency and purity grade of the proteins. In the present study, we propose for the first time the proof-of-concept of large-scale production process to obtain growth factors from plants as a new alternative to other productions systems. We have decided to select vascular endothelial growth factor (VEGF) as model assuming its key role in cell survival and regenerative medicine.

Several studies have demonstrated the benefit of exogenous VEGF application therapy on accelerating and enhancing tissue repair [13, 14]. It plays an important role in the stimulating epithelial and endothelial cells and acts specifically over the vascular system and it is considered as one of the strongest physiological regulators of angiogenesis [17-19]. In addition, VEGF may exert critical roles in tissue repair and regeneration regulating some key properties of stem cells. In fact, several types of VEGF receptors (VEGFRs) are highly expressed in the membrane of stem cells. VEGF induces the proliferation/migration, differentiation and growth-factor secretion of stem cells via activation of these receptors [20]. In the skin, VEGF secreted by adipose-derived stem cells (ASCs) mediates wound healing [21]. Furthermore, VEGF may be pivotal for the ASC-mediated improvement of hind-limb ischemia and myocardial infarction [22]. It is thought that VEGF stimulates ASCs via VEGF receptors in an autocrine manner. Additionally it has anti-apoptotic effects, increases angiogenesis and activates resident stem cells, which regenerate the damaged neighboring tissues in a paracrine manner.

Therefore, assuming its pivotal role in some critical steps of regenerative medicine and tissue regeneration, different biotechnological methodologies have been described to produce recombinant VEGF. Some of them include bacteria [24, 1], yeast [25], mammalian cells [2] and endosperm tissue of barley grain [6]. However, the study reported herein can be considered as the first transient expression of active recombinant human VEGF in *Nicotiana benthamiana* plants. Plants represent an attractive tool for some characteristics such as low production costs and the ability to synthesize proteins with post translational modifications, with a proper folding which ensures their biological activity. Furthermore, the expression in plants offers great advantages in biosafety considering that there is no risk of contamination with endotoxins or animal pathogens such as prions or virions [23].

Several human proteins have been produced using this plant virus-based expression vector including antimicrobials peptides, cytokines and antibodies [8]. One of the drawbacks of the system is the potential loss of stability of the recombinant vector [26], and evidences show that the size and nature of the included sequence may be involved. We evaluated the infectivity and stability of the VEGF recombinant vector during the infection process. Results from DAS-ELISA and RT-PCR demonstrate that our construction with human harmonized sequence of VEGF was extremely stable, even after 15 days post-inoculation. Thus high level of expression and accumulation of recombinant VEGF in plant cells was observed. This is in accordance with literature, proving that

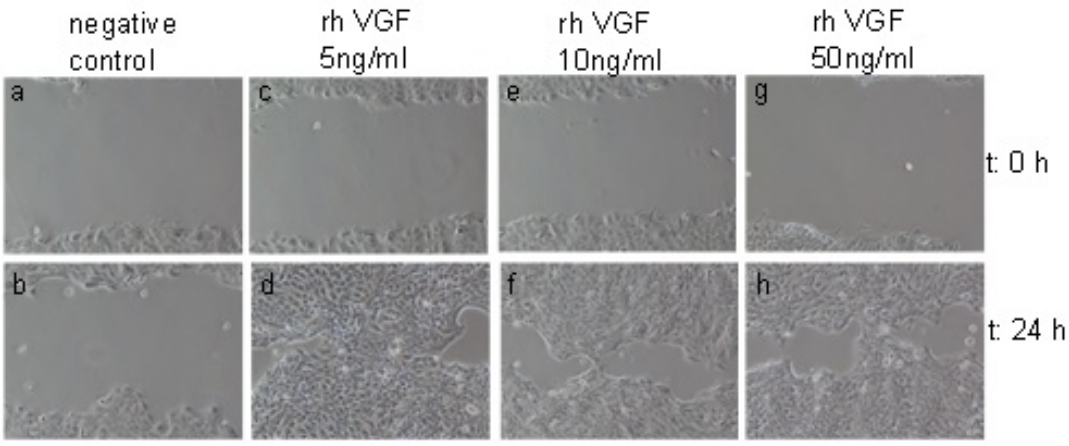

Fig. (6). Wound healing assay. **a-b**: negative control. **c-d**: 5 ng/mL rh VEGF. **e-f**: 10 ng/mL rh VEGF. **g-h**: 50 ng/mL rh VEGF. Upper: time 0; Lower: time 24 h.

transient expression allows recovering quite high amounts of recombinant protein in just a few weeks.

One of the most relevant issues talking of plant expression systems is the adjustment of a purification process that achieves high purity and efficacy levels good enough to an appropriate biological activity that fits market needs. The downstream purification process is the most challenging step in the plant expression system. We designed a protocol to recover high levels of active recombinant protein from plants. Only two-steps of purification are needed to obtain a final yield between 20-50 mg of human rh VEGF by kg of fresh tissue , with more than 97% of purity. Recombinant VEGF was accumulated as a glycosylated, disulfide-linked homodimeric protein with a molecular weight of 38-40 kDa. Correct folding and solubility of the recombinant protein was observed. The production of properly folded and biologically active recombinant VEGF in plants represents a major advantage over the E. Coli system where recombinant proteins often have to be refolded by denaturation / renaturation methods. The different rh VEGF sizes obtained corresponded to different levels of glycosylation because the presence of two motifs in the original sequence.

Several reports have documented the presence of endotoxins contamination in commercially available biomaterials and recombinant growth factors used for cell culture applications [6]. We have previously mentioned the advantages of plant systems in this regard. Recombinant VEGF produced in plant showed an endotoxin level less than <0.01EU per ug of protein, which could be considered suitable for *in vitro* and *in vivo* applications.

We demonstrated the biological activity of plant-derived rh VEGF by different assays. In the proliferation assay with Human Umbilical Vein Endothelial cells (HUVEC), we evaluated the dose-dependent stimulation of cell proliferation using a concentration range of 1.0-5.0 ng/mL of rh VEGF. Results indicated that rhVEGF exhibited more activity than prokaryotic-produced protein used as proliferation control. According to the action of VEGF in tissue repair, we used an *in vitro* "wound healing" model [27] to estimate the granulation tissue formation and re-epithelialization effects. Many studies suggest that local production of VEGF in the environment epithelial tissue has more significance than other growth factors accelerating wound-healing process [28].

Our results demonstrated the important role of plant-produced VEGF in skin repair and regeneration. In fact, it promotes *in vitro* fibroblast proliferation after the exogenous application and stimulates up to 100% skin cell regeneration in just 24hs after a wound.

In summary, this preliminary work highlights the power of plant used as biofactories to produce highly purified recombinant growth factors such as VEGF. The low endotoxin level and it biological potential to stimulate cell proliferation and wound healing could be exploited both in regenerative medicine (tissue repair and regeneration) and as a biological active ingredient in dermo-cosmetics.

CONCLUSIONS

We have developed an efficient and easy to scale up procedure for the purification of rh VEGF isoform 165 in *Nicotiana benthamiana* plants. Our procedure resulted in dimeric VEGF protein with high purity degree and yield, which showed full biological activity over endothelial and epithelial cells, confirming great potential for its use in regenerative medicine.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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REFERENCES

[1] Terpe, K. Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Appl. Microbiol. Biot.*, **2006**, *72*(2), 211-222.

[2] Zhu, J. Mammalian cell protein expression for biopharmaceutical production. *Biotechnol. Adv*., **2012**, *30*(5), 1158-1170.

[3] Shanmugaraj, B.M.; Ramalingam, S. Plant expression platform for the production of recombinant pharmaceutical proteins. *J. Biotechnol. Bioeng*., **2014**, *1*(6), 4.

[4] Ahmad, A.; Pereira, E.O.; Conley, A.J.; Richman, A.S.; Menassa, R. Green biofactories: recombinant protein production in plants. *Recent. Pat. Biotechnol.,* **2010***, 4*(3), 242-259.

[5] Ahmad, K. Molecular farming: Strategies, expression systems and bio-safety considerations. *Czech. J. Genet. Plant. Breed*., **2014**, *50*(1), 1-10.

[6] Magnusdottir, A; Vidarsson, H; Björnsson, J.M.; Örvar, B.L. Barley grains for the production of endotoxin-free growth factors. *Trends In Biotechnol.,* **2013***, 31*(10), 572-580.

[7] Horn, M.E.; Woodard, S.L.; Howard, J.A. Plant molecular farming: systems and products. *Plant. Cell. Rep*., **2004**, *22*(10), 711-20.

[8] Williams, L.; Deana, A.; Romero, A.; Molina, A.; Lunello, P. High-level production of active human TFPI-2 Kunitz domain in plant. *Protein Expr. Purif*., **2014**, *96*, 4-19.

[9] Gale, N.W.; Yancopoulos, G.D.; Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. *Genes Dev.*, **1999**, *13*(9), 1055-1066.

[10] Infanger, M.; Schmidt, O., Kossmehl, P.; Grad, S.; Ertel, W.; Grimm, D. Vascular endothelial growth factor serum level is strongly enhanced after burn injury and correlated with local and general tissue edema. *Burns*, **2004**, *30*, 305-311.

[11] Keck, P.J.; Hanser, S.D.; Krivi, G.; Sanzo, K.; Warren, T.; Feder, J.; Connolly, D.T. Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science*, **1989**, *246*(4935), 1309-1312.

[12] Tischer, E.; Mitchell, R.; Hartman, T.; Silva, M.; Gospodarowicz, D.; Fiddes, J.C.; Abraham, J.A. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J. Biol. Chem*., **1991**, *266*, 11947-11954.

[13] Bao, P.; Kodra, A.; Tomic-Canic, M.; Golinko, M.S.; Ehrlich, H.P.; Brem, H. The role of vascular endothelial growth factor in wound healing. *J. Surg. Res*., **2009**, *153*(2), 347-58.

[14] Galeano, M.; Deodato, B.; Altavilla, D.; Squadrito, G.; Seminara, P.; Marini, H.; Stagno d'Alcontres, F.; Colonna, M.; Calò, M.; Lo Cascio, P.; Torre, V.; Giacca, M.; Venuti, F.S.; Squadrito, F. Effect of recombinant adeno-associated virus vector-mediated vascular endothelial growth factor gene transfer on wound healing after burn injury. *Crit. Care. Med.,* **2003,** *31*(4), 1017-1025.

[15] Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **1970**, *227*(5259), 680-685.

[16] Towbin, H.; Staehelin, T.; Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci.,* **1979**, *76*(9), 4350-4354.

[17] Ferrara, N. Role of vascular endothelial growth factor in the regulation of angiogenesis*. Kidney Int.*, **1999**, *56*(3), 794-814.

[18] Detmar, M. The role of VEGF and thrombospondins in skin angiogenesis. *J. Dermat. Sci.,* **2000**, *24*(Suppl 1), 78-84.

[19] Ferrara, N. Role of vascular endothelial growth factor in regulation of physiological angiogenes. *Am. J. Physiol. Cell. Physiol.*, **2001**, *280*, 1358-1366.

[20] Bai, X.; Sadat, S.; Gehmert, S.; Alt, E.; Song, Y.H. VEGF receptor Flk-1 plays an important role in c-kit expression in adipose tissue derived stem cells. *FEBS Lett*., **2007**, *581*, 4681-4684.

[21] Lee, E.Y.; Xia, Y.; Kim, W.S.; Kim, M.H.; Kim, T.H.; Kim, K.J.; Park, B.S.; Sung, J.H. Hypoxia-enhanced wound-healing function of adipose-derived stem cells: increase in stem cell proliferation and up-regulation of VEGF and bFGF. *Wound Repair Regen*., **2009**, *17*(4), 540-547.

[22] Rehman, J.; Traktuev, D.; Li, J.; Merfeld-Clauss, S.; Temm-Grove, C.J.; Bovenkerk, J.E.; *et al.* Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation*, **2004**, *109*(10), 292-1298.

[23] Obembe, O.; Popoola, J.O.; Leelavathi, S.; Reddy, S.V. Advances in plant molecular farming. *Biotechnol. Adv.*, **2010**, *29*(2), 210-222.

[24] Siemeister, G.; Schnurr, B.; Mohrs, K.; Schachtele, C.; Marme, D.; Martiny-Baron, G. *Biochem. Bioph. Res. Co.*, **1996**, *222*(2), 249-255.

[25] Mohanraj, D.; Olson. T.; Ramakrishnan, S.A. Novel method to purify recombinant vascular endothelial growth factor (VEGF121) expressed in yeast. *Biochem. Bioph Res. Co*., **1995**, *215*(2), 750-756.

[26] Avesani, L.; Marconi, G.; Morandini, F.; Albertini, E.; Bruschetta, M.; Bortesi, L.; Pezzotti, M.; Porceddu, A. Stability of Potato virus X expression vectors is related to insert size: implications for replication models and risk assessment. *Transgenic Res.,* **2007***, 16*, 587-589.

[27] Singer, A.J.; Clark, R.A. Cutaneous wound healing. *N. Engl. J. Med.,* **1999**, *341*(10), 738-46.

[28] Ching, Y.H.; Sutton, T.L.; Pierpont, Y.N.; Robson, M.C.; Payne, W.G. The use of growth factors and other humoral agents to accelerate and enhance burn wound healing. *Eplasty*, **2011**, *11*, e41.

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