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Expression and Characterization of Human Vascular Endothelial Growth Factor Produced in SiHa Cells Transduced with Adenoviral Vector

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Abstract
The vascular endothelial growth factor (VEGF) is an essential factor to pathologic angiogenesis. Disruption of VEGF/VEGF receptor interaction in cancer patients inhibits the development of new and pre-existing tumor blood vessels. Consequently, VEGF becomes an important therapeutic target for handling solid tumors. In this work, human VEGF was produced in the culture supernatant of SiHa cells transduced with a replication-defective adenoviral vector (pAdhVEGF$_{121}$) encoding this molecule. The 35 kDa VEGF$_{121}$ homodimer was obtained from clarified culture media as a glycosylated protein. VEGF$_{121}$ expression levels were strictly dependent on the adenoviral viral load used. VEGF$_{121}$ was produced with purity over 98% after a single step chromatography by immobilized metal affinity chromatography. Additionally, VEGF$_{121}$ binds Bevacizumab antibody with a K_D of 7 nM. Biological characterization by mitogenic assay in HUVEC and ECV-304 cells showed that VEGF$_{121}$ stimulates cell proliferation in a dose-dependent manner in both cells. Finally, the neovascularization activity of VEGF$_{121}$ was demonstrated by vascular permeability assays in matrigel plug-bearing mice, showing significantly increased vasculature leakage after treatment with VEGF$_{121}$. Consequently, transduction of SiHa cells with adenovirus is a suitable alternative for manufacture heterologous proteins of therapeutic interest.

Keywords VEGF$_{121}$ · Adenoviral vector · HUVEC · ECV-304 cells · Vascular permeability

1 Introduction

The vascular endothelial growth factor-A (VEGF or VEGF-A) and its receptors play a fundamental role in angiogenesis. VEGF is a 40 kDa glycoprotein that is secreted to the extracellular compartment. The structure of VEGF is homodimeric, formed by two disulfide-linked monomers arranged in an antiparallel direction, displaying two binding sites at each extreme of the molecule [1]. In mammals, VEGF belongs to a family of five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF (Placental growth factor). The biological functions of VEGF proteins are mediated by a family of tyrosine kinase receptors, known as the VEGF receptors (VEGFR) [1–3]. The human VEGF-A gene is arranged in 8 exons and is expressed as multiple isoforms due to differential splicing. The four principal isoforms describe are hVEGF$_{121}$, hVEGF$_{165}$, hVEGF$_{189}$ and hVEGF$_{206}$ [4–7]. VEGF$_{121}$ lacks exons 6 and 7 responsible for the heparin-binding domain and freely diffuse through the extracellular matrix [5].

VEGF is a key regulator of vasculogenesis and angiogenesis during embryogenesis [8, 9]. However, in adults, physiological angiogenesis occurs only during wounds healing and the menstrual cycle [10–12]. When VEGF regulatory control is lost, angiogenesis contributes to numerous

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pathological conditions, such as rheumatoid arthritis [13] psoriasis [14], atherosclerosis [15], macular degeneration [16] and tumorigenesis [17].

VEGF induces cell proliferation and promotes survival and migration of endothelial cells. These biological effects are essential for tumorigenesis as angiogenesis is required for tumor growth and progression [17–20]. Cancer cells secrete VEGF promoting new blood vessels formation for nutrient and oxygen supply [21–23] Particularly, $VEGF_{121}$ isoforms overexpression has been detected in colorectal cancer and in a cancer mouse model $VEGF_{121}$ overexpression but not $VEGF_{165}$ overexpression induced tumor growth [24].

The interest of expressing biologically active $VEGF_{121}$ by recombinant DNA technology lies in the function of this molecule and its different uses: (i) To study and development of new therapies based on inhibiting VEGF/VEGFR interaction [25–29] and (ii) advanced treatments of patients with diabetic foot and ischemic diseases [30–36].

Recombinant VEGF has been expressed on different systems, including *Escherichia coli* [37–39], yeast [40, 41], insect cells [42] Chinese hamster ovary cells (CHO) [43]. Nevertheless, VEGF purification in these expression systems requires multiple-steps purification protocols increasing the final product cost [37, 44]. VEGF yields obtained on these systems are in the range of mg/l. However, VEGF expression has focused on $VEGF_{165}$ isoform.

In this work, we expressed $VEGF_{121}$ in SiHa cells transduced with adenoviral vectors. The use of replication-defective adenoviral vectors for transient gene expression of human proteins in mammal cell cultures is based on the fact that it is an efficient and safe method for gene transfer and protein expression in vitro and in vivo [45–51]. We analyzed the biological activity of purified $VEGF_{121}$ based on proliferation and induction of vascular permeability. $VEGF_{121}$ proliferative properties were analyzed on human umbilical cord endothelial cells (HUVEC) and ECV-304 cell line. The neoangiogenic activity was studied by in vivo murine model of vascular permeability.

2 Materials and Methods

2.1 Generation of Ad-VEGF Adenoviral Vector

The AdEasy vector system was used to generate the replication-defective vector $pAdhVEGF_{121}$ [52]. Briefly, a synthetic 500 bp sequence encoding human VEGF plus a 6xHis tag was cloned into *Xho*I/*Eco*RV sites of the shuttle vector pAdtrack-CMV. The resulting plasmid was transformed into the *E. coli* BJ5183 strain along with the pAdEasy vector to generate a defective adenoviral vector by in vivo recombination. The resulted plasmid ($pAdhVEGF_{121}$) has two cytomegaloviruses (CMV) promoters, one for the green fluorescent protein (GFP) and the other for VEGF. The initial viral pool was prepared by transiently transfecting the $pAdhVEGF_{121}$ plasmid into HEK-293 cells. Adenovirus stocks were further amplified in HEK-293 cells and harvested by a freeze-thaw lysis protocol. Virus concentration was calculated as gene transfer units (GTU) by GFP expression in HEK-293 cells.

2.2 $VEGF_{121}$ Production in SiHa Cells

SiHa cells were grown in a 24-well plate in DMEM medium supplemented with 10% fetal bovine serum (FBS). After reaching 80% of confluence, cells were infected with the replication-defective adenovirus at the following multiplicity of infection (MOI): 0.5, 10, 20, 40, 80, 160 GTU/cell. Six hours later, media was removed, cells were washed twice with PBS, and 2 ml of DMEM serum- free were added per well. After 72 h, supernatants were harvested, and VEGF expression was analyzed by SDS-PAGE, Western blot with an anti-Histidine antibody (Sigma, USA). $VEGF_{121}$ were quantified by the Novex^R Human VEGF solid-phase sandwich ELISA (ThermoFisher Scientific, EEUU).

2.3 $VEGF_{121}$ Purification from the Culture Medium

The expressed protein was purified by immobilized metal affinity chromatography (IMAC) using an ÄktaPurifier liquid chromatography system in the following manner. Cell culture supernatant was centrifuged and filtrated through a 0.2 µm filter to remove debris and floating cells. Clarified medium was applied to a 5 ml Ni-Sepharose fast flow column (GE, USA), previously equilibrated with 5 volumes of equilibrium buffer (0.5 mM PMSF, 100 mM NaH_2PO_4, imidazole 5 mM pH 7.4). After washing with equilibrium buffer containing 100 mM imidazole, the $VEGF_{121}$ protein was eluted by raising the imidazole concentration to 250 mM. Protein purification was monitored by UV absorption at A280 nm. Each collected fraction was analyzed by 12% SDS-PAGE/Coomassie staining. $VEGF_{121}$ was detected by Western blot with an anti-His antibody (Sigma, USA). The eluted fraction was dialyzed in 10 mM NaH_2PO_4, pH 7.4.

2.4 *N*-Deglycosylation of $VEGF_{121}$

The purified $VEGF_{121}$ was deglycosylated by PNGase F (New England Biolabs). $VEGF_{121}$ samples were mixed with denaturing glycoprotein buffer and incubated 5 min at 100 °C. The denatured $VEGF_{121}$ were incubated at 37 °C overnight with PNGase F in the buffer provided by the manufacturer and 1% NP40. VEGF was analyzed by SDS-PAGE/Coomassie blue staining and Western blot.

2.5 Anti-$VEGF_{121}$ Antibody Binding Affinity Measurement

The Monolith NT.115 device (NanoTemper Technologies GmbH, Germany) was used for analyzing binding affinity by microscale thermophoresis (MST). Bevacizumab antibody was labeled with NT-647 probe and used at a concentration of 6 nM. $VEGF_{121}$ was titrated from 3 pM to 100 nM. The experiments were performed in binding buffer (Tris–HCl 50 mM, pH 7.4, NaCl 150 mM, $MgCl_2$ 10 mM and 0.05% Tween-20). Samples were incubated for 1 h at room temperature before loaded into MST hydrophobic capillaries. The data were analyzed with NT Affinity Analysis software.

2.6 In Vitro Proliferation Assay

$VEGF_{121}$ biological activity was analyzed by MTT proliferation assays in Human umbilical vein endothelial cells (HUVEC) and ECV-304 cells. HUVEC cells were cultured in M-199 medium supplemented with 10% FBS and ECV-304 cells in DMEM medium supplemented with 10% FBS. HUVEC and ECV-304 cells were seeded in 96-well plates at 1×10^4 cells/well and 2×10^4 cells/well, respectively. After 24 h, cells were washed once with PBS and incubated for 6 h in serum-free medium before adding (0, 10, 20, 40, 80, 160, 320, 640 and 1280 ng/ml) of $VEGF_{121}$ for 48 h. Then, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at 5 mg/ml was added to each well and placed at 37 ° C for 4 h. Media were removed and formazan crystals were solubilized by adding 100 µl of DMSO. The plate was stirred for 15 min at 37 °C and measured the absorbance at 570 nm. EC50 value was calculated by the GraphPad Prism 6 software.

2.7 In Vivo Neovascularization Assay

$VEGF_{121}$ was mixed with Matrigel (BD Bioscience, USA) at 30 ng/ml and injected subcutaneously into the laterodorsal abdominal region of female CF1-BALB/c mice of 4 weeks of age. After 6 days, PEG IRDye 800CW contrast agent (LI-COR Biosciences, USA) was injected through the tail vein of Matrigel-$VEGF_{121}$ carrier mice. 24 h post-injection, the leakage of dye from capillaries was quantified by near-infrared fluorescent imaging (NIRF) using the Pearl® Impulse Small Animal Imaging System (LI-COR Biosciences, USA).

3 Results

3.1 VEGF Expression in Adenoviral-Transduced SiHa Cells

The replication-defective adenoviral vector pAdh$VEGF_{121}$ was constructed by cloning the synthetic $VEGF_{121}$ gene in pAdtrack followed by recombination with the pAdEasy vector in *E. coli* BJ5183 strain (Fig. 1a). pAdTrack-hVEGF and pAdEasy-hVEGF plasmids were digested with *Pac*I to check cloning and recombination events. A single band of 4.5 kbp in pAdEasy-hVEGF digested with *Pac*I indicated correct recombination between pAdTrack-hVEGF and pAdEasy (Fig. 1b). The recombinant viral genomes were transfected into the complementing cell line HEK-293. Replication-defective viruses manifest by comet-like fluorescent plaques around 7 days after transfection (Fig. 1c).

To determine $VEGF_{121}$ expression conditions, SiHa cells were infected at MOI of 5, 10, 20, 40, 80 and 160 GTU/ml. Transduction efficiency and cytopathic effect were evaluated by fluorescence microscopy (GFP expression) and phase contrast microscopy, respectively. Seventy-two hours post infection, the number, and fluorescent intensity of cells increased in a GTU dose-dependent manner; however, cytotoxicity and cell death also increased (Fig. 2a). For each MOI tested, we analyzed $VEGF_{121}$ expression by SDS-PAGE and Western blot, detecting two protein bands, at 17 kDa and at 19 kDa. A linear relationship was also observed between the amount of $VEGF_{121}$ secreted in the supernatants and the adenovirus load used to infect SiHa cells (Fig. 2b, c). Among 10–80 GTU/cell, the expression levels of $VEGF_{121}$ underwent an eightfold increase. Therefore, we select 80 UFC/cell for next experiments as the optimal MOI that allows a balance between the fewer cytopathic effect and increased $VEGF_{121}$ expression.

3.2 $VEGF_{121}$ Purification and Biochemical Characterization

The recombinant $VEGF_{121}$ was purified by IMAC. The chromatogram illustrates the elution profile of $VEGF_{121}$ on the IMAC column (Fig. 3a). Initial sample, non-binding fraction, washed fraction and eluted fraction were analyzed by 12% SDS-PAGE/Coomassie staining (Fig. 3b). $VEGF_{121}$ was purified as a homodimer of approximately 35 kDa with an estimated purity of 98% by densitometry with the Image J software. The protein was recognized by anti-poly-histidine antibody (Fig. 3c). 12 mg of purified $VEGF_{121}$ were obtained from 100 ml of culture medium using this purification procedure.

The glycosylation state of $VEGF_{121}$ was analyzed with PNGase F enzyme. We demonstrated that the upper band of 19 kDa detected on Western blots correspond to *N*-glycosylated forms of $VEGF_{121}$ monomers, whereas the lower band of 17 kDa contains non-*N*-glycosylated $VEGF_{121}$. Additionally, a third immunoreactive band of around 15 kDa was identified (Fig. 3d). Furthermore, it

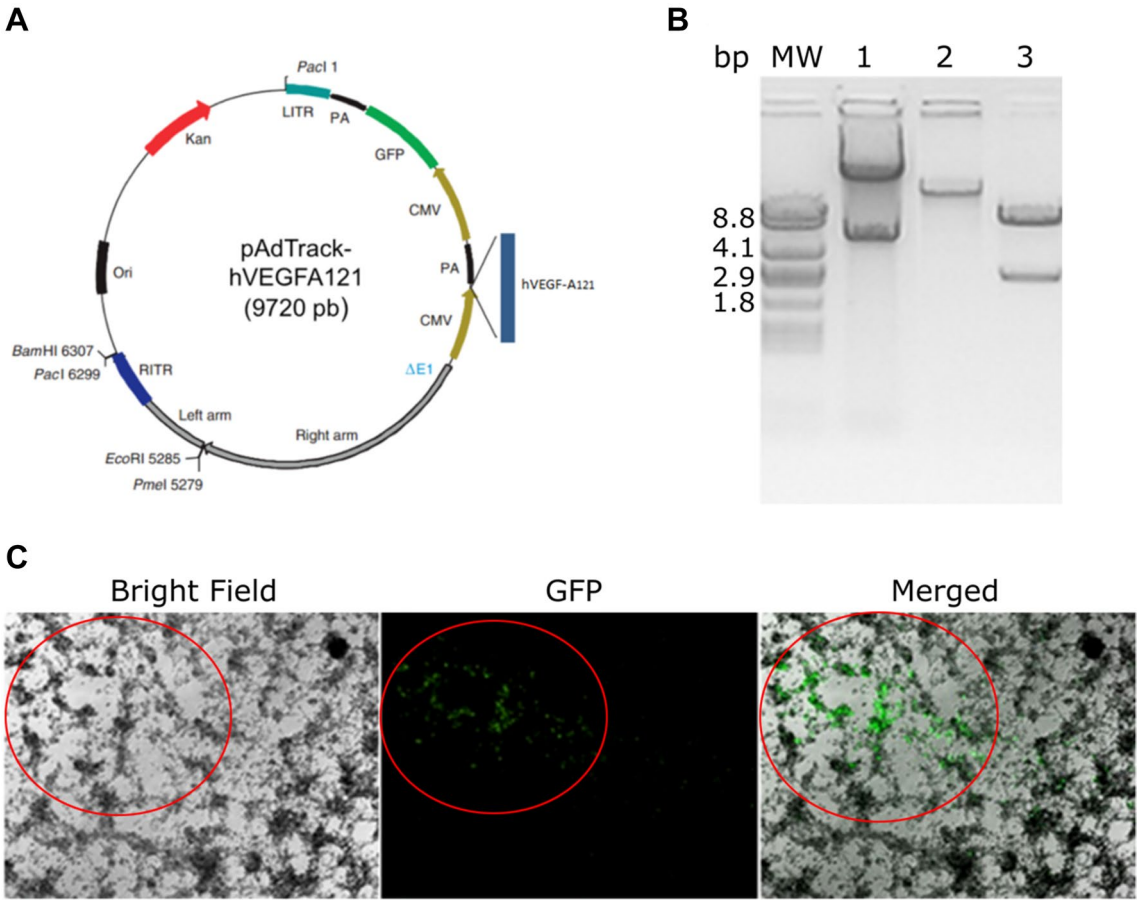

Fig. 1 Generation of replication-defective adenoviral vectors. **a** Construction of the recombinant adenoviral expression vector pAdTrack-hVEGF-A_{121}. A synthetic 500 bp band encoding human VEGF and a 6xHis tag was cloned in pAdtrack-CMV. **b** Restriction enzyme analysis of pAdTrack-hVEGF-A_{121} (Lane 1), pAdEasy (Lane 2), and homologous recombination product (Lane 3) with *Pac*I. **c** pAdhVEGF$_{121}$ was transfected in HEK-293 cells and GFP expression was visualized by fluorescence microscopy. Comet-like fluorescent plaques became apparent at 7 days (red circle). The microphotographs were acquired with an Olympus IX81 DSU microscope (×40 magnification)

was demonstrated by MST that VEGF$_{121}$ can bind Bevacizumab antibody with a K_D of 7 ± 1 nM (Fig. 4).

3.3 VEGF$_{121}$ Induces In Vitro Cell Proliferation in HUVEC and ECV-304 Cells

The biological activity of VEGF$_{121}$ was studied by its growth-stimulating effect on cells by MTT assays. The effect of recombinant VEGF$_{121}$ on HUVEC and ECV-304 cells proliferation was dose-dependent (Fig. 5a, b). An increase in VEGF$_{121}$ concentration caused HUVEC and ECV-304 cells increased proliferation. The half maximal effective concentration (EC50) of VEGF$_{121}$ to induce cell proliferation was estimated at 11.02 ng/ml and 13.03 ng/ml for HUVEC and ECV-304 cells, respectively.

3.4 VEGF$_{121}$ Increase In Vivo Neovascularization in Matrigel-Bearing Mice

VEGF$_{121}$ induced neovascularization was demonstrated by a matrigel plug assay in mice using IRDye® 800CW PEG as a contrast agent to visualize and quantify vascular permeability. NIRF-800 images showed that mice treated with VEGF$_{121}$ exhibited greater probe accumulation outside the vasculature than the placebo group, as a result of dye extravasation out of the matrigel plug (Fig. 6a, b).

4 Discussion

VEGF is a glycosylated protein that forms homodimers by interchain disulfide bonds. VEGF$_{121}$ homodimeric form is essential for its biological activity. VEGF is an asymmetric antiparallel homodimer with two receptor binding

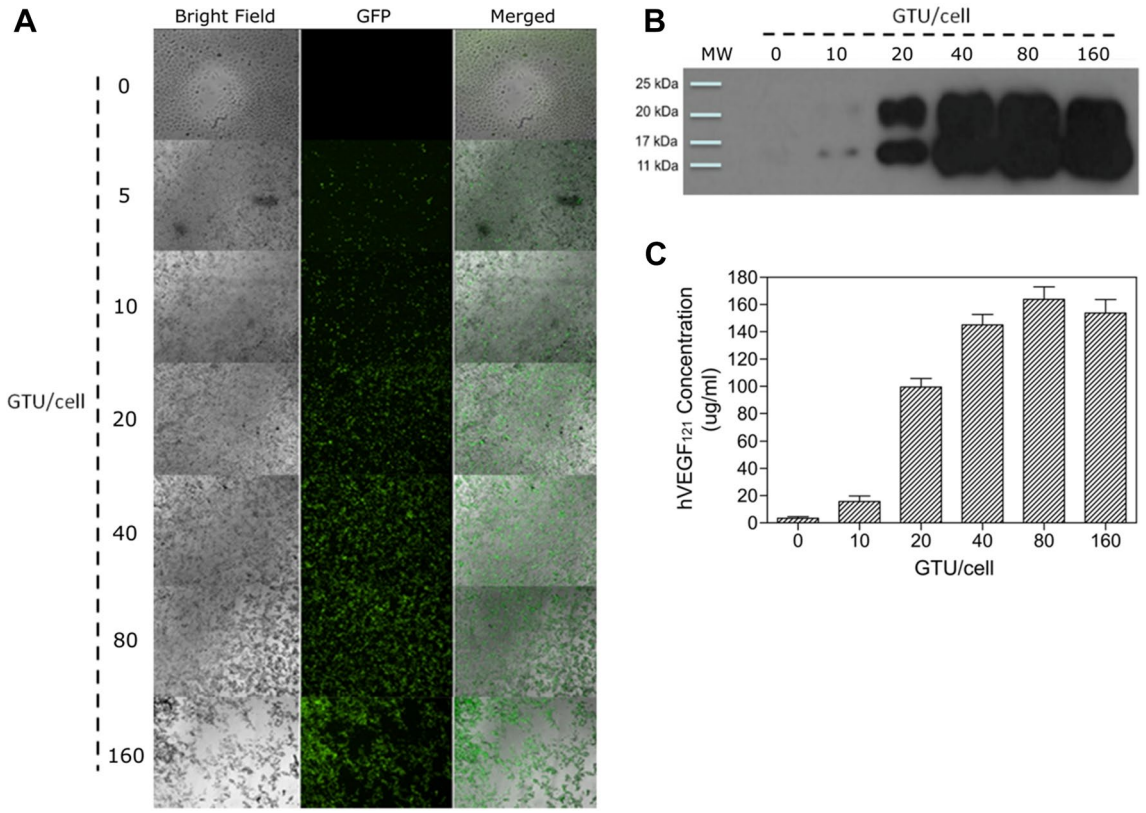

Fig. 2 Viral load effects on cytotoxicity and $VEGF_{121}$ expression. **a** SiHa cells were infected at MOI of 0, 10, 20, 40, 80 and 160 GTU/ml. Adenovirus infection resulted in a dose-dependent increase of cytopathic effect and GFP expression. **b** $VEGF_{121}$ expression in SiHa cells infected at different MOI. Proteins were separated in 12% SDS-PAGE gel under reducing conditions and immunodetected with an anti-Histidine antibody. At 20 MOI, two bands of $VEGF_{121}$ were detected, at 17 kDa and 19 kDa. However, at higher viral load $VEGF_{121}$ overexpression also increases giving rise to a saturation effect that affects the visualization of these 2 bands. **c** $VEGF_{121}$ expression levels at different viral load were quantified by Novex^{R} Human VEGF solid-phase sandwich ELISA

interfaces found at each pole of the homodimer. Each of the two binding interfaces must be able to contact a receptor monomer, triggering receptor dimerization and activation [1]. In the present work, we reported the expression of biologically active $VEGF_{121}$ by SiHa cells transduced with replication-defective adenovirus. We select this cell line because they express the Coxsackievirus/Adenovirus receptor (CAR) needed for effective adenoviral transduction [53–55]. Non-reducing SDS-PAGE/Western blot results demonstrated that $VEGF_{121}$ was secreted to the culture medium mainly as a homodimer with a near molecular weight of 35–37 kDa (58% of total protein, Fig. 3c, Lane 1). This result agrees with the molecular weight of $VEGF_{121}$ obtained from other sources, including yeast and bacteria [37, 40]. We did not detect monomer forms but higher-molecular-mass complexes (Fig. 3c, Lane 1). Expressions of $VEGF_{121}$ in *Pichia pastoris* and baculovirus/insect cells has also resulted in multiple VEGF bands, which was interpreted as covalently linked high-molecular-mass molecules [56, 57]. VEGF isoforms have been obtained in various conformations even in *E. coli*, with roughly equal proportions of monomeric and dimeric $VEGF_{121}$ forms [58].

Additionally, we demonstrated that $VEGF_{121}$ was secreted as a glycosylated protein. After *N*-deglycosylation with PNGase F enzyme, $VEGF_{121}$ migrated in SDS-PAGE as a band of around 17 kDa corresponding to the non-*N*-glycosylated $VEGF_{121}$ form. However, we detected a second immunoreactive band of approximately 15 kDa. We hypothesize that the 15 kDa band represent the *N*- and *O*-non-glycosylated form of $VEGF_{121}$ and the 17 kDa band represents the *N*-deglycosylated $VEGF_{121}$ that still contains O-linked oligosaccharides in two solvent-exposed serine residues (S74 and S95). Therefore, we believe that $VEGF_{121}$ is secreted as an *N*- and *O*-linked glycoprotein. While this hypothesis was not demonstrated, the presence of two accessible serine residues at position 74 and 95 in the molecular surface of $VEGF_{121}$ homodimer supports this hypothesis.

In this work, we did not perform a comparative analysis between glycosylated VEGF derived from SiHa cells and non-glycosylated variants. There is no evidence that VEGF glycosylation is required for dimerization neither binding to

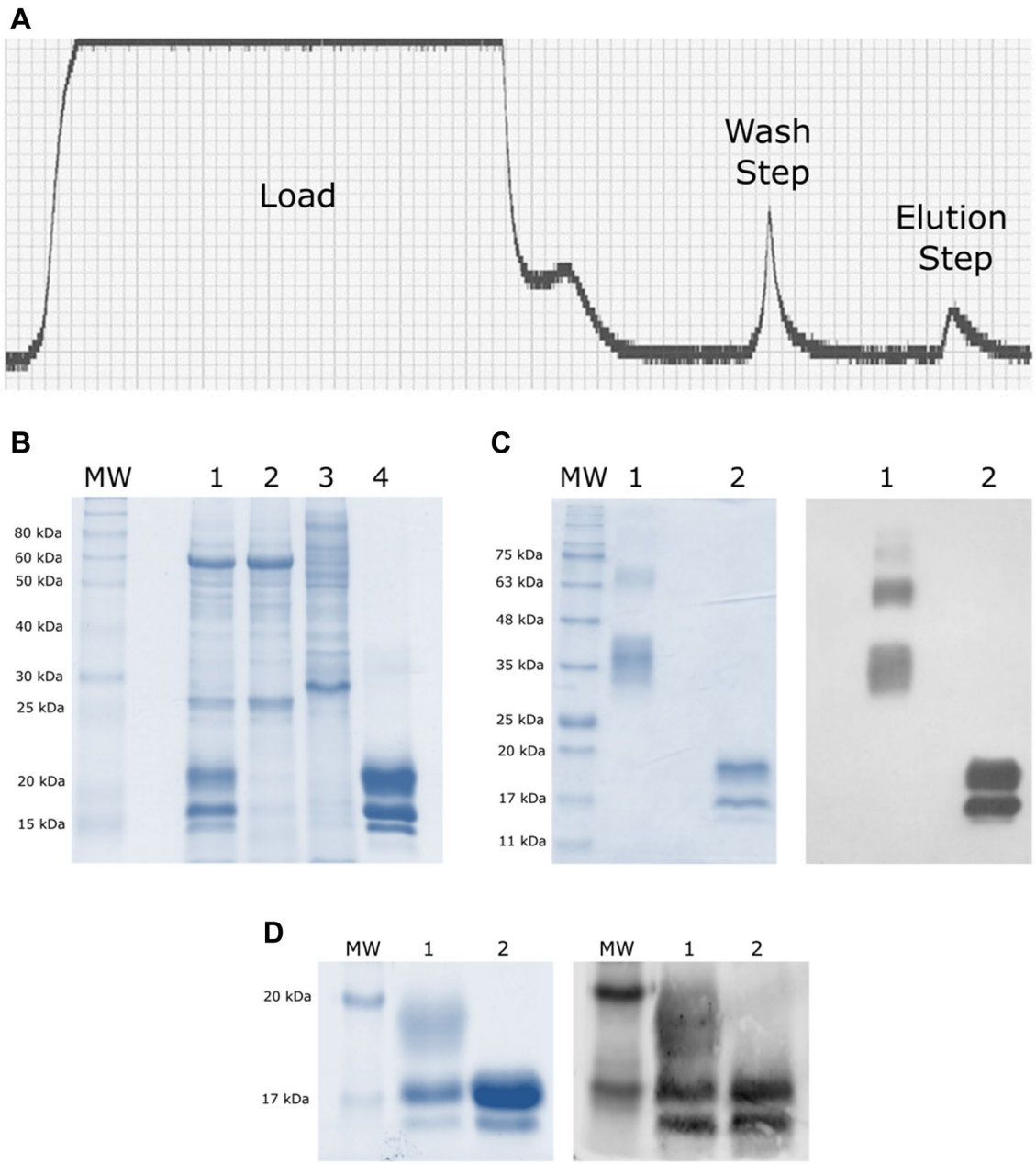

Fig. 3 Purification and characterization of $VEGF_{121}$. **a** Chromatogram of immobilized metal affinity chromatography using a Ni-Sepharose Fast Flow column. **b** SDS-PAGE analysis of samples from the affinity chromatography steps. Lane 1: Culture medium of SiHa cells (starting material); Lane 2: Flow-through; Lane 3: Proteins from the washing step at 100 mM Imidazole; Lane 4: $VEGF_{121}$ elution at 250 mM Imidazole. Bands were quantified by densitometry to estimate purity. **c** SDS-PAGE and Western blotting analysis of $VEGF_{121}$ produced in SiHa cells, respectively. Lane 1: Non-reducing conditions. Lane 2: Reducing conditions. **d** *N*-Deglycosylation assay of $VEGF_{121}$ by treatment with PNGase F enzyme. $VEGF_{121}$ (90 µg) was untreated (lane 1) or treated with PNGase F overnight (lane 2) at 37°C and analyzed by SDS-PAGE on a 12% gel under reducing conditions followed by Coomassie blue staining and Western blot with an anti-Histidine antibody

its receptor [59]. However, VEGF glycosylation is essential for molecule secretion; demonstrated by reduced secretion when glycosylation sites were mutated in mouse $VEGF_{164}$ and human $VEGF_{165}$ [59, 60]. Regarding its biological activity, it was demonstrated that VEGF glycosylation is not required for mitogenic activity [61]. Besides, in vessel permeability assays, there were no differences between VEGF with mutated glycosylation sites and wild-type VEGF [62].

$VEGF_{121}$ expressed in the supernatant of SiHa cells was purified by a single step of IMAC chromatography. We

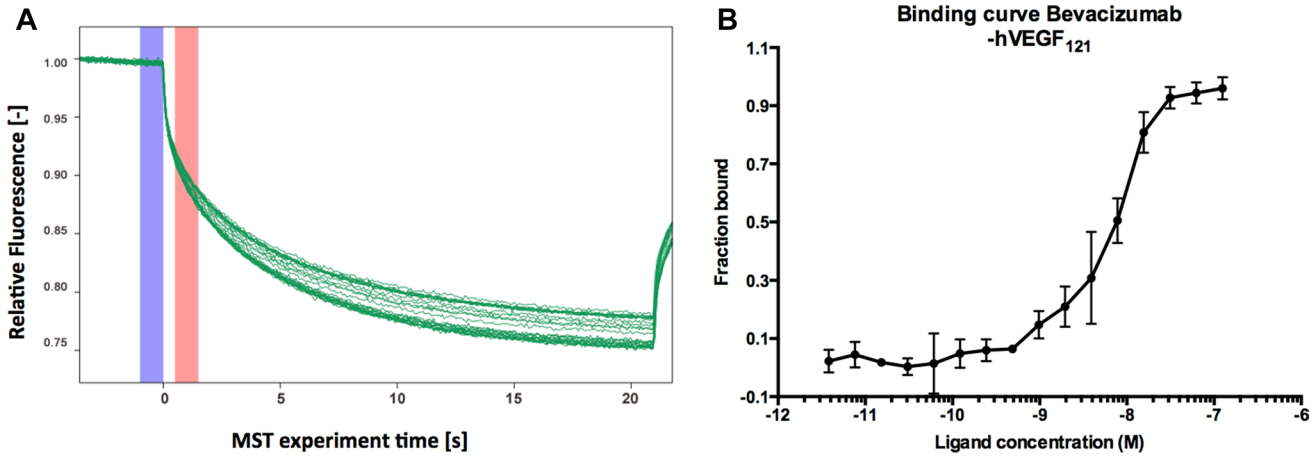

Fig. 4 The $VEGF_{121}$/Bevacizumab binding analyze by MST. **a** MST data for $VEGF_{121}$ interaction with Bevacizumab. **b** Binding curve between $VEGF_{121}$ and Bevacizumab. $VEGF_{121}$ at concentrations from 3 pM to 100 nM was titrated against 6 nM of Bevacizumab labeled with the fluorescent probe NT-650. MST experiments were performed at a LED power of 60%. Plotting of the change in thermophoresis and concomitant fitting of the data yielded a Kd of 7+ 1 nM. Error bars = s.d.; n = 2

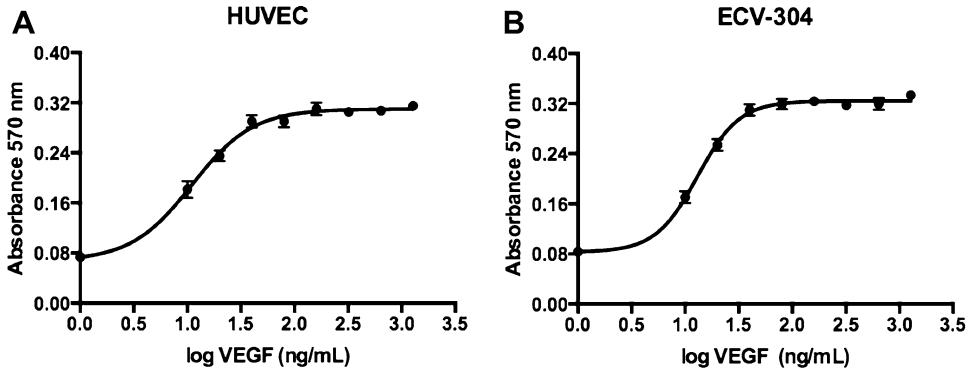

Fig. 5 Cell growth stimulation of HUVEC and ECV-304 cells by $VEGF_{121}$. HUVEC and ECV-304 cells were treated with increasing amounts of purified $VEGF_{121}$ (0, 10, 20, 40, 80, 160, 320, 640 and 1280 ng/ml), and cell proliferation was quantified by MTT assay. Error bars = s.d.; n = 3

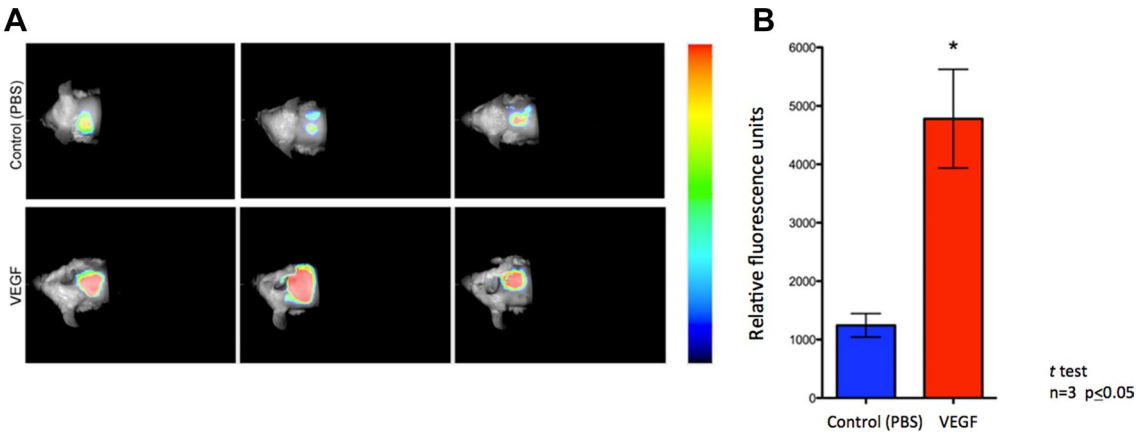

Fig. 6 $VEGF_{121}$-induces vascular permeability by matrigel plug assays.$VEGF_{121}$ (30 ng/ml) was mixed with matrigel and implanted on the back of CF1-BALB/c mice. The IRDye 800CW PEG probe was i.v injected 6 days later. Vascular permeability was measured by near-infrared fluorescence. **a** Representative NIRF-800 images of matrigel alone (upper panel) and matrigel-$VEGF_{121}$-bearing mice (lower panel) 24 h after NIRF dye inoculation. Extensive leakage of the fluorescent probe outside the matrigel plugs was observed on VEG_{121}-bearing mice. **b** Relative quantification of NIRF fluorescence of matrigel-$VEGF_{121}$-bearing mice. The statistical analysis was performed according to a *t*-test. Error bars = s.d; n = 3. Error bars = s.d; n = 3

obtained 12 mg of $VEGF_{121}$ from 100 ml of clarified culture media. The single-step purification method reported in this paper is simple, fast, and does not require difficult renaturation methods. $VEGF_{121}$ was expressed and purified from inclusion bodies in *E. coli* with high yield and purity [37, 63]. However, this procedure involves denaturation/renaturation steps. Conditions for refolding the denatured proteins to its native functional form are cumbersome and have to be standardized by trial-and-error methods, being time-consuming and expensive for scaling-up [64].

Bevacizumab is a therapeutic humanized antibody that blocks angiogenesis by inhibiting VEGF. This antibody recognizes a conformational epitope [1, 65], and bind to VEGF with a K_D of 4.45 nM [66]. By MST, we determine that Bevacizumab binds to the purified $VEGF_{121}$ with a K_D of 7 nM. The slight difference between the K_D determined by MST and reported K_D for Bevacizumab, seem to be a consequence of different experimental conditions. However, this result confirms that the $VEGF_{121}$ expressed in SiHa cells transduced with adenoviral vector keep the conformational epitope recognized by Bevacizumab, and suggest that $VEGF_{121}$ is secreted properly folded.

VEGF has two major biological activities: (a) it is mitogenic to vascular endothelial cells and (b) can induce capillary leakage (vascular permeability) [67]. VEGF stimulates HUVEC proliferation and promotes its migration. Therefore, HUVEC cells are generally used for assaying VEGF biological activity [68, 69]. $VEGF_{121}$ produced in SiHa cells was able to induce mitogenic responses in HUVEC in a dose-dependent manner. The EC_{50} of $VEGF_{121}$ was 11.02 ng/ml, similar to the EC_{50} reported to VEGF purified from bacteria and yeast [37, 40]. Although HUVEC is the most extensively used endothelial cell type to study endothelial functions [68] and responds to VEGF, these cells are isolated as primary cell culture. Working with primary cultures is complex, and it is only possible to keep them in vitro for short periods of time, besides there are increased risks of microorganisms' contamination. Thus, working with a cell line [70] that has endothelial properties could replace the use of HUVEC.

ECV-304 cell line was first described as a spontaneously transformed cell line originated from a Japanese human umbilical vein endothelial cell culture. Later, it was found to be derived from the human urinary bladder carcinoma T24 cell line [71]. However, ECV-304 cells have many features of endothelial cells [72], such as increasing cell proliferation in the presence of VEGF [73], a characteristic only observed in endothelial cells but not found in T24 cells [74]. Hence, ECV-304 cells would serve as an angiogenesis model allowing us to characterize endothelial functions [75]. Based on this information, we evaluated the mitogenic activity of $VEGF_{121}$ on ECV-304 cells. $VEGF_{121}$ produced in SiHa cells was able to enhance ECV-304 cell proliferation in a cell dose-dependent manner, with an EC_{50} of 13.03 ng/ml. This result suggests that ECV-304 cell can be used to evaluate the biological activity of $VEGF_{121}$, in replace of HUVEC primary cell culture.

VEGF induced neovascularization is characterized by immature and highly fenestrated blood vessels. There is a direct relationship between neovascularization and vascular permeability. Classically, neovascularization has been measured using the Miles Assay [76]. This test uses a spectrophotometer to quantify the leakage of a dye from the vasculature to the surrounding tissue, with the limitation of the analysis of a single time point, which must be selected empirically. In this work, we present a modified method to visualize and quantify in real-time the vascular leakage using a fluorescent dextran-based dye. $VEGF_{121}$ mixed in the matrigel plug injected into mice induced the formation of new and highly fenestrated blood vessels, which trigger the leakage of the fluorescent probe from the vasculature to the plugs. Thus, $VEGF_{121}$ caused an increase in fluorescence accumulation in matrigel plugs.

5 Conclusions

In this study, we demonstrated functional expression of $VEGF_{121}$ in the culture media of SiHa cells transduced with a replication-defective adenoviral vector (pAdhVEGF121). $VEGF_{121}$ expression levels were strictly depended on the viral load used, reaching the maximum expression level at 80 GTU/cell. Expression in SiHa cells resulted in a soluble *N*-glycosylated dimer of 35–37 kDa, and partial formation of high molecular weight oligomers covalently linked. The purified $VEGF_{121}$ was recognized by the anti-VEGF antibody Bevacizumab, demonstrating that $VEGF_{121}$ produced in SiHa cells keep the conformational epitope recognized by Bevacizumab. $VEGF_{121}$ induced cell proliferation in both HUVEC and ECV-304 cell line. Therefore, ECV-304 cells can replace the primary culture model HUVEC for assaying VEGF bioactivity. Finally, a vascular permeability test with mice carrying matrigel plugs and a fluorescent probe made it possible to determine that purified $VEGF_{121}$ promotes capillary leakage. In conclusion, we have successfully developed an easy and efficient procedure for $VEGF_{121}$ expression and purification. This molecule could be used for the evaluation of therapies against angiogenic tumors and wound healing.

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