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Isoform of Vascular Endothelial Cell Growth Inhibitor ($VEGI_{72–251}$) Increases Interleukin-2 Production by Activation of T Lymphocytes

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Abstract The objective of this study is to investigate the characteristics of the recombinant variant of human vascular endothelial cell growth inhibitor, $VEGI_{72–251}$, and compare its biological activities with that of its prototype $VEGI_{24–174}$. The recombinant plasmid containing the variant $VEGI_{72–251}$ gene was constructed and expressed in *Escherichia coli*. The effects of the expressed $VEGI_{72–251}$ on cell proliferations were checked in the human umbilical vein endothelial cell line and certain tumor cell lines (ECV304 and B16). The inhibition of $VEGI_{72–251}$ on angiogenesis was detected in the chorioallantoic membrane of chick embryos. In comparison with $VEGI_{24–174}$, the recombinant human $VEGI_{72–251}$ seems to have no effect on the proliferation of endothelial cells and the angiogenesis of the chorioallantoic membrane *in vitro*. An enzyme-linked immunosorbent assay-based method was used for the measurement of interleukin-2 (IL-2) production by peripheral blood monocytes (PBMCs) treated with $VEGI_{72–251}$. PBMCs were pretreated with $VEGI_{72–251}$ (1.25−12.50 μg/ml) for 24 h *in vitro*, and the IL-2 concentration in PBMC medium was increased from 354 pg/ml to 1256 pg/ml. It can be concluded that $VEGI_{72–251}$ is able to increase the level of human IL-2 production by the activation of T lymphocytes. Differing from $VEGI_{24–174}$ on anti-angiogenesis, $VEGI_{72–251}$ may serve as an anti-cancer factor through its activation of T lymphocytes.

Key words vascular endothelial cell growth inhibitor; biological activity; interleukin-2

Vascular endothelial cell growth inhibitor (VEGI) was first identified from a human umbilical vein endothelial cell (HUVEC) cDNA library by Tan *et al.* in 1997 [1]. The full gene of VEGI is approximately 17 kb, which consists of I, II, III and IV exons and three introns. The VEGI gene can produce three kinds of mRNA, which are translated into three VEGI isoforms consisting of 251, 192 and 174 amino acid residues, respectively [2,3]. Recombinant VEGI comprising only the putative extracellular domain was shown to be an effective inhibitor of endothelial cell proliferation in culture [2]. $VEGI_{251}$ is encoded by I, II, IIIb and IV exons, and $VEGI_{174}$ is encoded by IVa and IVb exons. Each isoform has the IVb exon and the 151 residue domain at the C-terminal shared by all the isoforms [4].

The protein $VEGI_{174}$ has been well studied, but little is known about $VEGI_{251}$. $VEGI_{251}$ (also named TL1A) is the most abundant of the three isoforms. Similar to most tumor necrosis factor family members, hydrophobicity analysis predicts $VEGI_{251}$ to be a type II membrane-bound protein with residues 72–251 comprising the extracellular domain (named $VEGI_{72–251}$). Previous studies showed that recombinant $VEGI_{72–251}$, from *Escherichia coli*, exerted potent activity on activating T cells but had no significant anti-angiogenic activity *in vitro* or *in vivo* [3]. But Chew and his colleagues found that $VEGI_{251}$ accounted for its anti-angiogenic activity *in vivo* [4]. We have previously studied the biological activity of $VEGI_{24–174}$ and the relationship between its structure and biological activities [5,6].

In order to determine the biological activity of $VEGI_{251}$ and the difference between $VEGI_{24–174}$ and $VEGI_{72–251}$, we cloned and expressed the soluble $VEGI_{72–251}$ and investigated its biological activities.

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Materials and Methods

Materials

Endonucleases *Eco*RI and *Bam*HI, T4 DNA ligase, phytohemagglutinin, fetal calf serum, RPMI 1640 medium and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco BRL (Grand Island, USA). Primers were synthesized by Sangon Bioengineering (Shanghai, China). The upstream primer was 5′-AGGGAATTC-ATGCTAAAAGGACAGGAGTTTG-3′ and the downstream primer was 5′-CAGGGATCCCTATAGTAAGAAG-GCTCCA-3′. Cloning vector pMD18-T, expression vector pBV220 and *E. coli* DH5α were routinely stored in our laboratory. The extraction kit for a small amount of plasmid, acrylamide, the RNA extraction kit, protein molecular weight marker and NBT/BCIP were purchased from Shanghai Huashun Bioengineering (Shanghai, China). ECV304, HUVEC and B16 cells were maintained in our laboratory. *Taq* enzyme was purchased from Sangon Bioengineering. Embryonated eggs were purchased from the Second Military Medical University Laboratory Animal Center (Shanghai, China). N,N,N′,N′-tetramethylethylenediamine (TEMED) and β-mercaptoethanol were purchased from Janssen Pharmaceutica (Beerse, Belgium). Sephacryl S-200 and DEAE Sepharose were obtained from Pharmacia (Uppsala, Sweden). Rabbit anti-$VEGI_{24–174}$ polyclonal antibody was prepared by this laboratory, and the goat anti-rabbit immunoglobulin G-AP was purchased from Huamei Biotechnology (Shanghai, China). Human interleukin (IL)-2 Minikit was purchased from Endogen (Woburn, USA).

Gene cloning of $VEGI_{72–251}$

The HUVEC cells were cultured and the total RNA was isolated from the cells by Trizol methods. Reverse transcription was used to reverse-transcript the first strand of cDNA using 0.1 μg RNA as the template and the *Taq* enzyme was employed for the following polymerase chain reaction (PCR). The gene fragment was amplified by PCR: 32 cycles of 94 ºC for 30 s, 58 ºC for 30 s, 72 ºC for 45 s; and 72 ºC for 6 min. The 540 bp fragment ($VEGI_{72–251}$ cDNA) with restricted endonuclease *Eco*RI and *Bam*HI sites was cloned from the total RNA of HUVECs.

Plasmid construction

PCR products were inserted into pMD18-T vector and transformed to host strain *E. coli* DH5α. The plasmid was extracted from the host strain and digested with *Eco*RI and *Bam*HI, then identified by DNA sequencing. A 540 bp fragment was recovered, inserted into the same site of pBV220 and transformed to *E. coli* DH5α. The recombinant expression plasmid was then extracted. The constructed plasmid was identified by restriction enzyme analysis.

Expression and purification of soluble $VEGI_{72–251}$

The confirmed recombinant positive DH5α colony was incubated at 42 ºC for 4 h. Bacteria were collected by centrifugation (10 min, 2500 *g*) and followed by sonication. The inclusion bodies were washed in buffer (2 M urea, 0.1 M Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl), and refold in buffer (50 mM KH_2PO_4, pH 8.0, 1 mM EDTA, pH 8.0, 2 mM H-GST, 1 mM O-GST). The refolded protein $VEGI_{72–251}$ was dialyzed against phosphate-buffered saline (PBS) for 48 h and the supernatant of the suspension was separated by centrifugation. The supernatant was purified by the DEAE (10 cm×2.5 cm, 50 mM PBS, pH 6.0, 10 mM NaCl equilibrated, 0.01–1 M NaCl linear gradient elution). The component of each elution peak was collected. Then the elution was purified by the Sephacryl S-200 (60.0 cm×1.5 cm, 50 mM PBS eluted), and analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Protein analysis

Molecular weight identification of the soluble $VEGI_{72–251}$ was detected by SDS-PAGE. Purity of the recombinant protein was analyzed by gelatin scan analysis system (Pharmacia-LKB, St. Albans, UK).

Identification of the expressed proteins by Western blot analysis

$VEGI_{72–251}$ and $VEGI_{24–174}$ were transferred to nitrocellulose membranes after SDS-PAGE, then the proteins were detected with the rabbit anti-$VEGI_{24–174}$ polyclonal antibody.

Cell proliferation assay

HUVEC, ECV304 and B16 cells were seeded in 96-well plates at a density of $1×10^4$ cells per well in DMEM or RPMI 1640 medium with or without 10% fetal bovine serum. The cells were cultured at 37 ºC with 5% CO_2 for 24 h, then changed with fresh medium. The purified $VEGI_{72–251}$ and $VEGI_{24–174}$ were added, with gradually increasing concentrations, and incubated at 37 ºC for 48 h. Cell growth inhibition was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Effect of $VEGI_{72–251}$ on chorioallantoic membrane (CAM)

A hole of approximately 1 cm diameter was bored into the eggshell at the narrow apex after 9 d of incubation.

The CAM was exposed after the eggshell's membrane was torn using the injection needle. A small methylcellulose saucer was placed on the CAM where there were few blood vessels, then the protein solutions (5 μg of $VEGI_{24–174}$, 5–20 μg of $VEGI_{72–251}$ and PBS) were added into the saucer. The chick embryos were incubated in constant humidity at 37.8 ºC. After 3 d incubation, the CAMs were examined.

$VEGI_{72–251}$ stimulates peripheral blood monocytes (PBMCs) to secrete IL-2

PBMCs isolated from whole blood were adjusted to 5×10^6 PBMCs/ml in RPMI 1640 supplemented with 10% fetal calf serum, 65 μg/ml of phytohemagglutinin and $VEGI_{72–251}$. The $VEGI_{72–251}$ (25 μg/ml) was diluted in series and the absence of $VEGI_{72–251}$ was the control. After PBMCs were incubated for 24 h in 24-well tissue culture plates, 200 μl supernatant was harvested from each well. The supernatants from the duplicate wells were mixed, numbered and stored at −20 ºC. IL-2 was tested, strictly following the manufacturer's instructions.

Results

Cloning and identification of $VEGI_{72–251}$ gene

The cloned $VEGI_{72–251}$ fragment was inserted into pDM18-T vector. The plasmid was extracted from the host strain and digested with *Eco*RI/*Bam*HI. A 540 bp fragment and the vector fragment were recovered. The 540 bp fragment sequence was identical to that recorded in GenBank. The recombinant plasmid pBV220-$VEGI_{72–251}$ was identified by enzyme digestion (*Eco*RI/*Bam*HI), and corresponding fragments were obtained [**Fig. 1(A)**]. The recombinant plasmid pBV220-$VEGI_{72–251}$ was transformed to *E. coli* DH5α.

Expression and purification of recombinant protein

Expression plasmid (pBV220-$VEGI_{72–251}$) of the recombinant protein was induced at 42 ºC for 1–6 h. An obvious band of approximately 20 kDa was observed by SDS-PAGE after induction, which matched the predicted molecular weight of 20 kDa. The influence of induction time on the expression level was analyzed by SDS-PAGE. The results showed that the expression products appeared as early as the first hour and reached a peak in the fourth hour. Densitometric scanning showed that this band constituted more than 25.6% of total bacterial protein. After sonication, $VEGI_{72–251}$ was almost exclusively found in the insoluble fraction. Insoluble $VEGI_{72–251}$ was solubilized, refolded, dialyzed and purified by DEAE and Sephacryl S-200. Purified protein appeared as a fairly homogenous band of 20 kDa on SDS-PAGE. Densitometric scanning showed that the purified protein band constituted more than 94.5% [**Fig. 1(B)**].

Western blot analysis of soluble $VEGI_{72–251}$ and $VEGI_{24–174}$

It was found that the recombinant $VEGI_{72–251}$ could cross-react with the rabbit anti-$VEGI_{24–174}$ polyclonal antibody as well as $VEGI_{24–174}$ [**Fig. 1(C)**]. This result indicated that $VEGI_{24–174}$ and $VEGI_{72–251}$ have the same

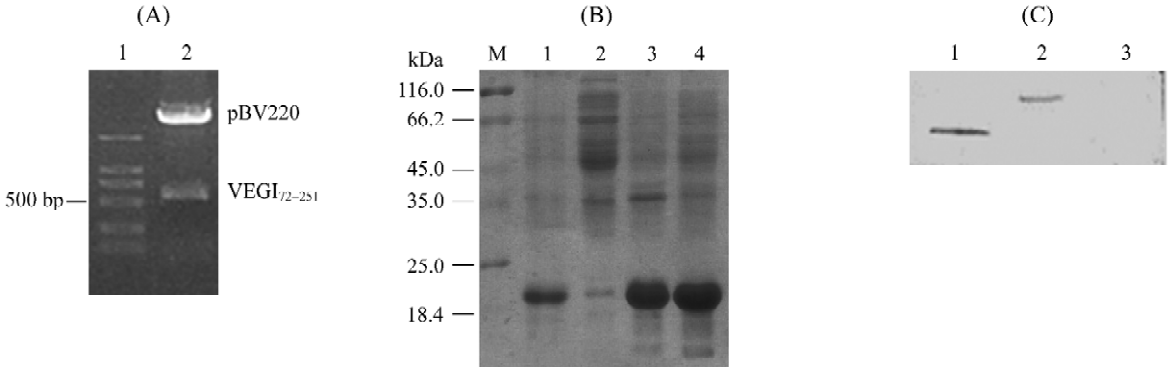

Fig. 1 Identification of recombinant plasmid pBV220-$VEGI_{72–251}$ and its detection using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

(A) Identification of pBV220-$VEGI_{72–251}$. 1, DL2000 DNA marker; 2, recombinant pBV220 digested with *Eco*RI/*Bam*HI. (B) Detection of $VEGI_{72–251}$ with SDS-PAGE. M, standard protein marker; 1, total protein in pellet after sonication; 2, total protein in supernatant after sonication; 3, DEAE purified $VEGI_{72–251}$; 4, Sephacryl S-200 purified $VEGI_{72–251}$. (C) Detection of $VEGI_{72–251}$ with Western blot analysis. 1, $VEGI_{24–174}$; 2, $VEGI_{72–251}$; 3, negative control.

epitope and the 151 residues at the C-terminal at least have an epitope which can interact with the rabbit anti-$VEGI_{24–174}$.

Biological activity of soluble $VEGI_{72–251}$ on HUVEC and tumor cells

HUVEC, ECV304 and B16 cells were cultured in the presence of different concentrations of $VEGI_{72–251}$ (0–80 μg/ml) for 72 h. MTT assay was used to detect the effects of soluble $VEGI_{72–251}$ on proliferation activity of HUVECs and tumor cells. There was no distinct effect on HUVEC or tumor cell growth inhibition [**Fig. 2(A)**].

Effect of $VEGI_{72–251}$ on angiogenesis of CAM

We found that there was no obvious inhibitory effect on proliferation of the blood vessels of CAMs after treatment with 1–20 μg $VEGI_{72–251}$, and no visible difference between $VEGI_{72–251}$ and PBS. But the number of blood vessels was decreased and the diameter of the vessels was attenuated after the CAMs were treated with 5 μg $VEGI_{24–174}$ [**Fig. 2(B)**].

Detection of IL-2 in supernatants of PBMC stimulated by $VEGI_{72–251}$

T cells secrete IL-2, and IL-2 is one of the most important growth factors of T cells. The contents of IL-2 secreted by T cells could be detected in the culture medium. We found that $VEGI_{72–251}$ could stimulate the T cells treated with 1.56–12.5 μg/ml $VEGI_{72–251}$ for 24 h to secrete IL-2 [**Fig. 2(C)**].

Discussion

VEGI has three isoforms and the isoform $VEGI_{174}$ has been well studied [7]. In 1999, Zhai and his colleagues reported that 1–25 residues at the N-terminal of $VEGI_{174}$

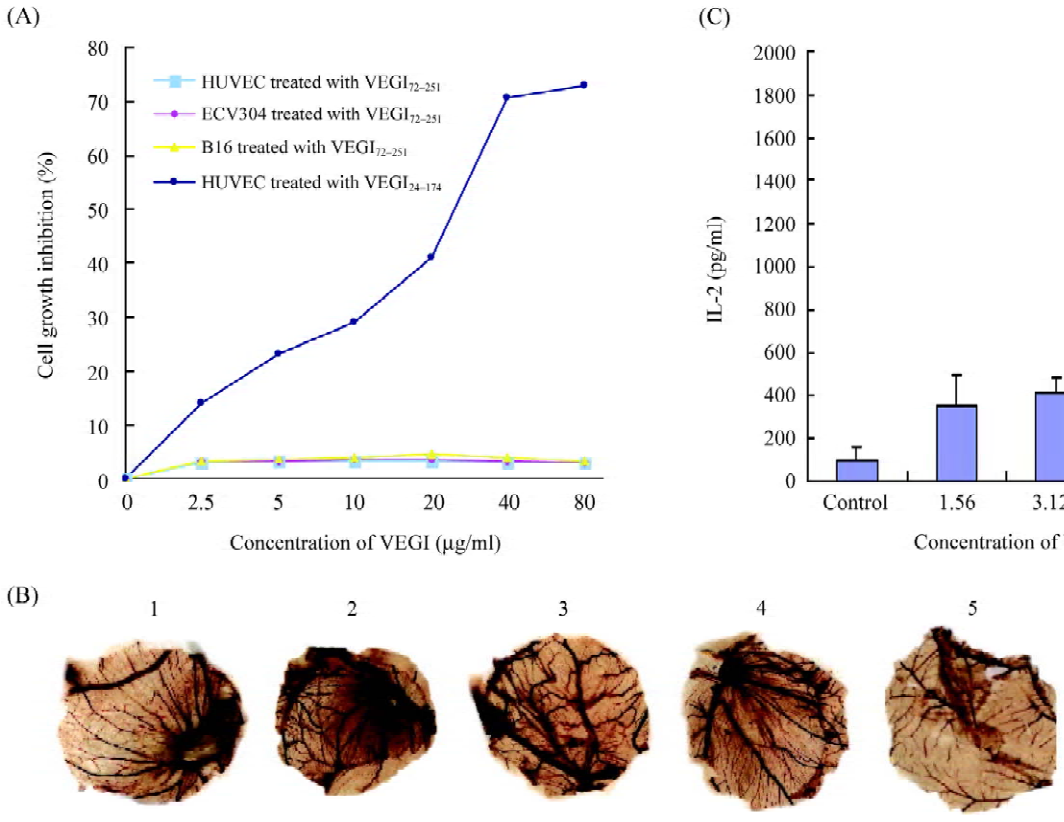

Fig. 2 Biological activities of vascular endothelial cell growth inhibitor (VEGI)

(A) Inhibitory effect of VEGI on the growth of human umbilical vein endothelial cells (HUVEC), ECV304 and B16 cells. The cells were treated with VEGI (0–80 μg/ml) for 72 h and the growth inhibition was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (B) Effect of $VEGI_{72–251}$ and $VEGI_{24–174}$ on chorioallantoic membrane angiogenesis. 1, phosphate-buffered saline; 2, 5 μg $VEGI_{72–251}$; 3, 10 μg $VEGI_{72–251}$; 4, 20 μg $VEGI_{72–251}$; 5, 5 μg $VEGI_{24–174}$. (C) Secretion of interleukin (IL)-2 from peripheral blood monocytes stimulated with $VEGI_{72–251}$. Control, RPMI 1640 containing 12.5 μg/ml $VEGI_{24–174}$.

were the predicted intracellular and transmembrane domain [2]. The 26–174 residues at the C-terminal, an extracellular domain, made up the soluble protein. This protein can markedly inhibit the growth of endothelial cells but not other types of examined cells, such as T cells [8]. It could not only activate JNK and P38MAPK, but also inhibit formation of capillary-like structures by endothelial cells [9–11]. *In vivo*, a secreted form of VEGI significantly inhibited the growth of colon carcinoma in mice and the growth of breast cancer xenograft tumors [12]. However, overexpression of full-length $VEGI_{174}$ in cancer cells was ineffective on tumor growth. These observations indicate that recombinant VEGI comprising only the putative extracellular domain was shown to be an effective inhibitor of endothelial cell proliferation in culture [2,12].

In order to study the biological activity of $VEGI_{251}$ and the difference between $VEGI_{174}$ and $VEGI_{251}$, the extracellular domain of $VEGI_{251}$, $VEGI_{72–251}$, was cloned and expressed in *E. coli* DH5α. We found that solubility of the recombinant soluble protein reached 0.9 g/L. It indicated that the solubility of this protein is high. Analysis by DNA tools showed that the recombinant protein's isoelectric point was 6.6 and its predicted molecular weight was 20 kDa. It was confirmed by SDS-PAGE that the molecular weight was approximately 20 kDa. Western blot analysis found that the rabbit anti-$VEGI_{24–174}$ polyclonal antibody could detect the $VEGI_{72–251}$ legibly, which indicated that $VEGI_{24–174}$ and $VEGI_{72–251}$ have the same epitope and the 151 residues at the C-terminal at least have an epitope which can interact with the rabbit anti-$VEGI_{24–174}$. Although the 151 residues at the C-terminal of $VEGI_{72–251}$ are identified with $VEGI_{24–174}$, the assay of biological activity found that $VEGI_{72–251}$ did not possess the biological activities of $VEGI_{24–174}$, such as inhibiting the growth of endothelial cells. It was also found that $VEGI_{72–251}$ has the biological activities of increasing IL-2 production by activation of T cells. These results indicated that the structure binding with the T cell receptors lies at the N-terminal of $VEGI_{72–251}$, and $VEGI_{24–174}$ can not bind with T cells. The possible reason is that soluble $VEGI_{72–251}$ is not able to bind with endothelial cells, which might be due to a change of protein structure or its structure at the C-terminal covering the structure binding with endothelial receptors. All of these speculations should be tested by further studies.

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