

## Research Article

# Preparation and Characterization of a Novel Chimeric Protein VEGI-CTT in *Escherichia coli*

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Vascular endothelial cell growth inhibitor (VEGI) is a recently identified antiangiogenic cytokine that belongs to the TNF superfamily, and could effectively inhibit endothelial cell proliferation and angiogenesis. Synthetic peptide CTT (CTTHWGFTLC) has been found to suppress invasion and migration of both tumor and endothelial cells by potent and selective inhibition of MMP-2 and MMP-9. To prepare chimeric protein VEGI-CTT for more potent antitumor therapy, the recombinant expression vector pET-VEGI-CTT was constructed. This fusion protein was expressed in inclusion bodies in *E. coli* BL21 (DE3), and was refolded and purified by immobilized metal affinity chromatography using His-tag. Purified VEGI-CTT protein was characterized by proliferation assays of the endothelial cells and casein degradation assay in vitro. The results demonstrated that chimeric protein VEGI-CTT had a potent activity of antiangiogenesis through inhibiting the proliferation of endothelial cells, and could effectively reduce the activity of MMP-2 and MMP-9. The preliminarily in vivo study demonstrated that chimeric protein VEGI-CTT had more potent antitumor activity than VEGI and/or CTT peptide against CA46 human lymphoma xenografts in nude mice. Thus, these facts that are derived from the present study suggest that the chimeric protein VEGI-CTT may be used for tumor therapy in the future.

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## 1. INTRODUCTION

The success of applying an antiangiogenic agent Avastin [1] in clinical settings for cancer treatment provided the first set of evidence to support the hypothesis that inhibition of tumor neovascularization can bring significant benefit to cancer therapy [2]. Because neovascularization under either physiologic or pathologic conditions is controlled by balance of endogenous proangiogenic and antiangiogenic factors, an important approach to develop therapeutic agents for cancers and other angiogenesis-driven diseases is to use endogenous antiangiogenic factors [3].

Vascular endothelial growth inhibitor (VEGI), also known as tumor necrosis factor superfamily (member 15 (TNFSF15) and TL1A), is a recently identified antiangiogenic cytokine [4–6]. The VEGI gene is expressed predominantly in endothelial cells, and the VEGI mRNA is detectable in many adult human organs, suggesting a physiologic role for this unique gene in the maintenance of the normal vasculature [7]. Previous reports showed that VEGI is a potent and specific inhibitor of endothelial cell growth [4–7]. There are three differential splicing isoforms: VEGI-251, VEGI-192,

and VEGI-174 [8–10]. The initially reported VEGI protein is composed of 174 amino acids [4, 5]. Hydrophobic analysis predicted VEGI-174 to be a type II transmembrane protein, similar to most tumor necrosis factor (TNF) family members [9]. Recombinant VEGI comprising only the putative extracellular domain exhibited effective inhibition of endothelial cell growth but had no effect on the proliferation of breast tumor cells or smooth muscle cells [4]. Full-length VEGI-174 was found, however, to have no effect on tumor growth when overexpressed in cancer cells [5], whereas a secretable fusion protein (sVEGI) comprising a secretion signal peptide and the putative extracellular domain of VEGI-174 inhibited tumor growth when overexpressed in cancer cells [5]. This indicates that a solubilized extracellular domain of VEGI is responsible for its biological activity.

Several lines of evidence suggested that tumor growth, angiogenesis, and metastasis are dependent on matrix metalloproteinase (MMP) activity. The two MMPs most closely correlated with metastasis potential are the 72 kDa MMP-2 (gelatinase A) and the 92 kDa MMP-9 (gelatinase B) [11, 12]. The synthetic CTTHWGFTLC-deca peptide (CTT peptide) has been recently described as specific and selective

inhibitor for MMP-2 and -9, which belongs to a novel class of cyclic tissue-permeable peptides containing an HWGF motif, and could not inhibit other MMPs or gelatinolytic serine proteinases [13, 14]. CTT peptide has been evidenced to suppress invasion and migration of both tumor and endothelial cells in vitro, prevent the growth and invasion of tumors in mice, and specifically home to tumor vasculature expressing gelatinases (MMP-2 and -9) in vivo.

The objective of our study was to prepare chimeric protein VEGI (extracellular domain)-CTT for more potent antitumor therapy. In the present study, we successfully expressed and purified recombinant protein VEGI-CTT in *E. coli*. The purified VEGI-CTT showed inhibition of endothelial cell proliferation and formation of capillary-like tubes by ABAE cells. In addition, the chimeric protein VEGI-CTT potently reduced the activity of MMP-2 and MMP-9. The preliminarily in vivo study demonstrated that chimeric protein VEGI-CTT had more potent antitumor activity than VEGI or CTT peptide against CA46 human lymphoma xenografts in nude mice.

The results described here suggest that bacterial-expressed VEGI-CTT could permit further study on its inhibitory activity of tumor growth, invasion or metastasis, molecular mechanism, and potential application for antiangiogenesis therapy of tumor clinically.

## 2. MATERIALS AND METHODS

### 2.1. Materials and cell lines

NIH 3T3 cells, lymphoma cell line CA46, and liver cancer cell line HepG2 were purchased from the American Type Culture Collections (ATCC, Rockville, Md, USA). Adult bovine aortic endothelial (ABAE) cells, human umbilical cord vein endothelial (HUVE) cells, and human vascular smooth muscle (HVSM) cells were purchased from Clonetics (San Diego, Calif, USA). All cells were maintained in their recommended medium.

Chelating Sepharose Fast Flow and Superdex75 resin were obtained from Amersham Pharmacia Biotech (USA), and isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was purchased from Sigma-Aldrich (USA). The expression vector pET30a(+), which contains a T7 promoter, His-Tag sequence, and an ampicillin resistance gene, and bacterial strains BL21(DE3) were purchased from Merck Biosciences (Germany). The plasmid pSW200 which contains full-length VEGI was a kind gift from Dr. Jingjuan Yao. The PCR reagents and the molecular biology enzymes were purchased from Takara Bio Inc. (Japan). BCA protein assay reagent was from Pierce Biotechnology (Ill, USA). CTTHWGFTLC-deca peptide was chemically synthesized. Recombinant VEGI protein was previously generated by our lab and used as control in the present study.

### 2.2. Construction of prokaryotic expression vector pET-VEGI-CTT

A prokaryotic expression vector pET-VEGI-CTT was constructed by subcloning the fragment containing the VEGI-CTT gene. The recombinant cDNA sequence encoding

VEGI-CTT was amplified by PCR from the original plasmid pSW200 containing full-length VEGI cDNA using a forward primer P1: 5'-TCCTCTAGAATGTGTACAACCTCAGTGGGGTTTCACACTTTGCGTGAGACAAACTCCCACA-3' and a reverse primer P2: 5'-CGCCTCGAGCTATAGTAAGAAGGCTCC-3'. The forward primer contained an Xba I site followed by CTT (CTTHWGFTLC) sequence and the N-terminal sequence of VEGI extracellular domain. The reverse primer possessed a complementary sequence of the C-terminus of VEGI extracellular domain, a stop codon, and an Xho I site. The final PCR product was directly cloned into the pGEM-T vector and then subjected to DNA sequencing to verify the desired sequence. PCR conditions consisted of 30 cycles of 94°C for 0.5 minute, 55°C for 0.5 minute, and 72°C for 1.5 minutes. The PCR-amplified DNA fragment was separated on 1% agarose gels containing ethidium bromide, and then subjected to DNA sequencing to verify the desired sequence. The amplified cDNA fragment was subcloned into *E. coli* expression vector pET30a(+) to construct the prokaryotic expression vector pET-VEGI-CTT (Figure 1).

### 2.3. Expression and purification of chimeric protein

*E. coli* BL21(DE3) was transformed with expression plasmid pET-VEGI-CTT. Bacterial cultures were incubated at 37°C in LB growth medium with antibiotic selection (100 ng/mL ampicillin), and grown until early log phase ( $A_{600} = 0.4-0.8$ ). The targeted protein expression was induced at 37°C by the addition of IPTG to a final concentration of 1  $\mu$ M, and incubated with shaking for 2–8 hours. The bacteria were collected by centrifugation at 4000 rpm for 30 minutes.

The pellet was resuspended in 20  $\mu$ M Tris-HCl (pH 8), sonicated at maximal output, and centrifuged at 12000 rpm for 30 minutes at 4°C. The pellet was an inclusion body which was denatured by 8 M urea for 2 hours at room temperature, then centrifuged at 10000 rpm for 30 minutes at 4°C, and the supernatant was filtered (0.45  $\mu$ M pores) and loaded at room temperature onto an His-Ni<sup>2+</sup> metal affinity column pre-equilibrated with 8 M urea, 100  $\mu$ M NaH<sub>2</sub>PO<sub>4</sub>, 10  $\mu$ M Tris, and 10  $\mu$ M imidazole. Refolding of the bound protein is performed by the use of a linear 8–0 M urea gradient, starting with the equilibrating buffer above and finishing with 50 column volumes of equilibrating buffer without urea, then eluted with 5 column volumes of eluting buffer (20  $\mu$ M phosphate, 500  $\mu$ M NaCl, and 300  $\mu$ M imidazole). Protein concentration was determined by bicinchoninic acid (BCA) assay.

### 2.4. SDS-PAGE and Western blotting

To confirm and compare the quality of recombinant protein expression, 10–12  $\mu$ L from each fraction were analyzed by SDS-PAGE (12% gels) followed by staining with Coomassie brilliant blue R-250. For western blotting analysis, proteins were electrotransferred to PVDF (Amersham-Pharmacia Biotech) membranes using Mini Trans-Blot cell (Bio-Rad) following manufacturer's instruction, blocked in 0.5% BSA in TBS (100  $\mu$ M Tris, 0.9% NaCl, pH 7.5), and immunoreacted with a 1 : 5000 dilution of mouse anti-His monoclonal

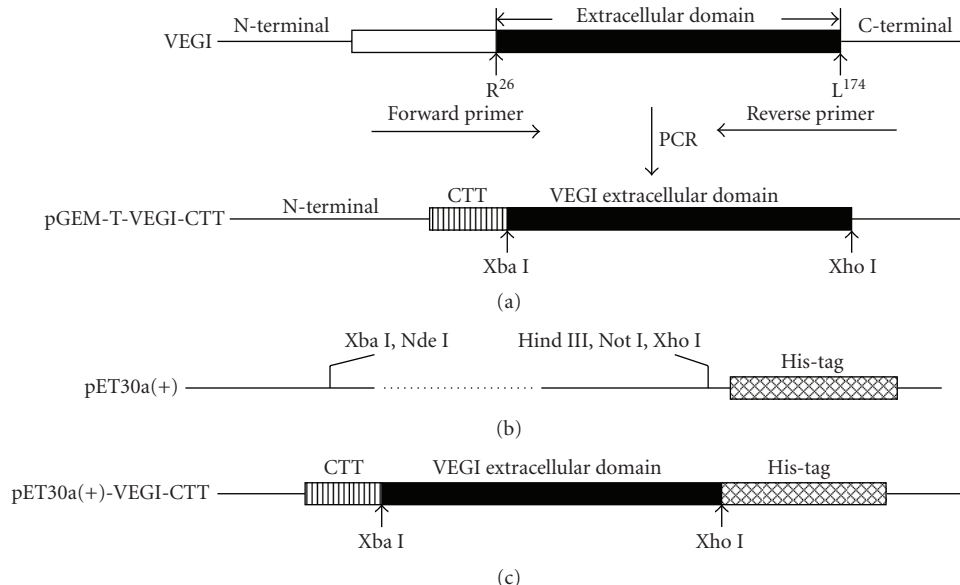


FIGURE 1: Schematic representation of the cloning steps of VEGI-CTT fragment into the expression vector pET30a(+). (a) The vector Psw200 containing full-length VEGI gene was used to amplify recombinant cDNA of human VEGI-CTT for 483 bp by PCR. The recombinant cDNA of human VEGI-CTT was cloned into pGEM-T, and produced pGEM-T-VEGI-CTT. (b) pET30a(+) vector with His-tag upstream of the multiple cloning site that contains XbaI and XhoI. (c) pET30a(+) carrying XbaI-XhoI fragment of VEGI-CTT to produce pET30a(+)-VEGI-CTT. The resulting fusion gene contains internal His-tag and VEGI-CTT.

antibody (TIANGEN, Beijing, China), followed by an HRP-labeled goat antimouse IgG (Zymed, USA). Chromogenic-based detection was performed using DAB as a staining substrate.

## 2.5. Cell proliferation assay

Cells were seeded in triplicate at 3000–5000/well in 96-well plates in IMEM (GIBCO, Gaithersburg, Md, USA) containing 10% fetal calf serum (FCS), and cultured at 37°C with 5% CO<sub>2</sub>. ABAE and HUVE cell culture media contained additional 1 ng/mL and 6 ng/mL FGF-2, respectively. VEGI-CTT, VEGI, or CTT peptide in different concentrations was added to each well, and the blank control wells were only added with equal volume of PBS. The media were changed once on day 3. The number of viable cells was determined on day 6 by using a Coulter (Hialeah, Fla, USA) counter.

## 2.6. In vitro angiogenesis assay

Quantitative assessment of capillary-like tube formation by endothelial cells cultured on collagen gels was carried out as described previously

[4]. To prepare a three-dimensional collagen gel, 0.5 mL chilled IMEM with 10% FBS and 0.7 mg/mL rat tail type I collagen (BD, Franklin Lakes, NJ, USA) was added to each well of a 24-well plate. After formation of the collagen gel (about 1–2 mm thickness), ABAE cells were seeded at  $5 \times 10^4$  cells/well. The cultures were maintained in IMEM with 10% FCS, 1 ng/mL of FGF-2, 5% CO<sub>2</sub>, and 37°C for 72 hours. The media were replaced with fresh media omitting FGF-2, and were cultured for 48 hours. Then the media were replaced

with fresh media containing 20 ng/mL of FGF-2. The cultures were maintained at 37°C for 48 hours. The gels were then fixed with cold methanol. The abundance of the capillary-like structures formed by ABAE cells was determined by computer-assisted image analysis. The relative intensity of the capillary-like structures was measured as the ratio of the total length of the tubular structures over the total areas measured (mm/mm<sup>2</sup>).

## 2.7. In vitro $\beta$ -casein degradation assay

Inhibition of MMP-9 and MMP-2 by the chimeric protein VEGI-CTT was measured using casein degradation assay (13). Subsequently, MMP-2 (2.5  $\mu$ g) or MMP-9 (2.5  $\mu$ g) was run on a 10% SDS-PAGE containing 2 mg/mL  $\beta$ -casein. The gel was first washed in Triton X-100 containing buffer to remove SDS, and it was cut into slices that were immersed into the solutions containing different concentrations of VEGI-CTT, VEGI, or CTT peptide. After incubation for 48 hours at 37°C, the gels were stained with Coomassie Blue and scanned, and the digested areas were quantitated using image analysis (Global Lab Image 3.2; Data Translation Inc. and Acuity Imaging Inc., Marlboro, Mass, USA).

## 2.8. In vivo study

Six- to eight-week-old female nude mice were obtained from the Planned Parenthood Research Institute, Shanghai, China. All animals in this study were housed under pathogen-free conditions and were maintained in accordance with guidelines of the Committee on Animals of the Second Military Medical University, Shanghai, China.

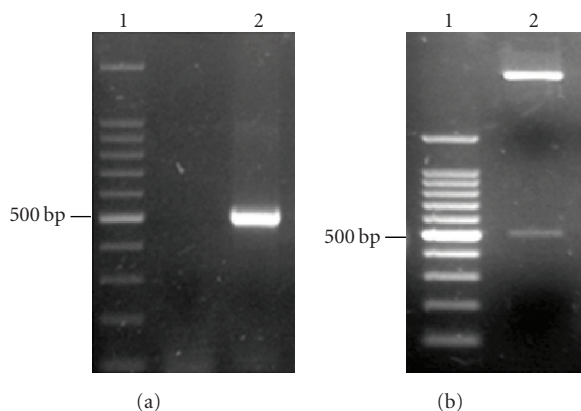


FIGURE 2: Construction of prokaryotic expression vector pET-VEGI-CTT. (a) DNA fragments of VEGI-CTT were amplified by PCR. Lane 1: 100 bp markers. Lane 2: VEGI-CTT fragment of 486 bp. (b) Restriction enzyme analysis of the prokaryotic expression vector pET-VEGI-CTT. Lane 1: 100 bp markers; lane 2: pET-VEGI-CTT digested by Xba I and Xho I.

The xenograft CA46 model was carried out as described [15]. In brief, xenografts were allowed to be established to an average size of 50–100 mm<sup>3</sup>, after which mice were randomized into various conditional groups. VEGI-CTT protein, VEGI protein, or CTT peptide was given to each mouse at designated dose via intraperitoneal injection at a frequency of twice a week. Each mouse was measured for tumor size using a caliper on alternate days. Animal body weight and any sign of morbidity were also closely monitored. The treatment lasted for 2 weeks; at predesigned points mice were killed, and tumor xenografts were extirpated, weighed, and correlated with the tumor size measurement.

### 2.9. Statistical analysis

Between-group differences were determined using a Student's *t*-test, and multiple treatment groups were compared within individual experiments by analysis of variance or the Kruskal-Wallis test. *P* values less than .05 were considered to be statistically significant.

## 3. RESULTS

### 3.1. Construction of prokaryotic expression vector pET-VEGI-CTT

The vector pSW200 containing full-length VEGI gene was used to amplify recombinant cDNA of human VEGI-CTT by PCR. Analysis of 1% agarose gels revealed that the resulting fragment was ≈490 bp (Figure 2(a)), and then VEGI-CTT cDNA was cloned into pGEM-T vector for sequencing, which showed a fragment with 486 bp in length.

The amplified cDNA fragment was subcloned into pET30a(+) to produce a prokaryotic expression vector pET-VEGI-CTT; subsequently the resulting pET-VEGI-CTT was identified by digestion of restriction enzymes XbaI and

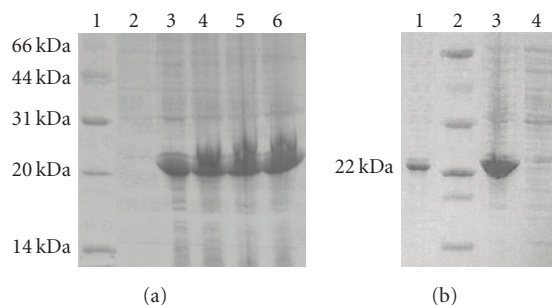


FIGURE 3: Expression analysis of recombinant VEGI-CTT in BL21(DE3). (a) Time course analysis of recombinant VEGI-CTT induction in BL21(DE3) induced by 1 μM IPTG. Protein samples were prepared from the inclusion-body fraction of induced bacterial cells at time points of 2 hours (lane 3), 4 hours (lane 4), 6 hours (lane 5), and 8 hours (lane 6) of the induction, and were resolved on 12% SDS-PAGE. Lane 1: low molecular weight protein markers; lane 2: uninduced control. No difference was found from lane 3 to lane 6 at 2–8 hours. (b) SDS-PAGE analysis of expression and location of the fusion protein VEGI-CTT in the cellular fractions of *E. coli*. Lane 1: eluted fraction under renaturing conditions with 500 μM imidazole; lane 2: low molecular weight protein markers; lane 3: the inclusion-body fraction; lane 4: supernatant of cell lysates.

XhoI, which revealed that the cDNA of VEGI-CTT was correctly inserted into the vector pET30a(+) (Figure 2(b)).

### 3.2. Expression, refolding, and purification of chimeric protein VEGI-CTT

The resulting plasmids pET30a(+)-VEGI-CTT and the controls were transformed into the *E. coli* BL21(DE3). This construction of pET30a(+)-VEGI-CTT should produce a recombinant protein of approximately 22 kDa. Induction of the strong T7 promoter controlling the expression of VEGI-CTT in BL21(DE3) with 1 μM IPTG produced recombinant VEGI-CTT after 2 hours. It seemed that there was no difference in expression levels from 2 to 6 hours at 37°C (Figure 3(a)). The recombinant protein is the major band present at all time points of induction.

To determine the location of the recombinant VEGI-CTT in the cellular fractions of *E. coli*, the cytosolic, inclusion-body, periplasmic, and media fractions were tested. The recombinant protein was found only in the inclusion-body fraction; almost no VEGI-CTT protein was observed in the soluble fraction (Figure 3(b)). Likewise, no indication of secreted or transferred protein was observed in the media or periplasmic fractions. No effect on the solubility of the recombinant protein was observed by decreasing the incubation temperature or by slowing the expression by decreasing the concentration of the IPTG (results are not shown).

Following the successful overproduction of recombinant VEGI-CTT in the inclusion-body fraction of *E. coli*, we attempted to use the presence of the internal His-tag in the fusion protein for renaturation and purification on immobilized metal ion-affinity chromatography (Ni<sup>2+</sup>-chelating) column. The recombinant VEGI-CTT in inclusion body was

TABLE 1: Purification of chimeric protein VEGI-CTT from inclusion bodies (from 1 L of *E. coli* culture and 4.6 g wet weight cells).

Steps	Total protein (mg)	Step yield (%)	Overall yield (%)
Cell lysate	304	—	—
Inclusion body	155	100	100
Solubilization (denaturation buffer containing 8 M urea)	96.3	62.1	62.1
Refolding and affinity purification	25.5	26.6	16.5

denatured by 8 M urea. A step-down urea concentration strategy (a linear gradient from 8 to 0 M urea) was applied to the VEGI-CTT on-column refolding process. The VEGI-CTT fusion proteins were eluted with 300  $\mu$ M imidazole, with purity being greater than 90%, and they were of the expected 22 kDa (Figure 3(b)). By this strategy, a stable final amount of the obtained purified recombinant VEGI-CTT was approximately 25.5 mg/L of initial bacterial broth. The refolding and purification yield of recombinant VEGI-CTT was about 16.5% (Table 1).

To insure that the recombinant protein we had expressed and renatured was the protein we designed, the VEGI-CTT was subjected to western blotting analysis using His-tag monoclonal antibody. As shown in Figure 4, the purified recombinant protein is His-immunoreactive and, as expected, it has a molecular weight of approximately 22 kDa.

### 3.3. Chimeric protein VEGI-CTT inhibits the proliferation of the endothelial cells in vitro

The bioactivity of VEGI-CTT was determined by the proliferation of ABAE and HUVE cells in vitro. As shown in Figure 5(a), the protein was found to preferentially inhibit the FGF-2-induced proliferation of ABAE and HUVE cells in a dose-dependent manner, whereas it could not inhibit the growth of HVSM, liver cancer HepG2 or NIH 3T3 cells. The half-maximum inhibitory concentrations ( $IC_{50}$ ) of VEGI-CTT for ABAE and HUVE cells were about 25 ng/mL and 100 ng/mL, respectively. The inhibition effect of VEGI-CTT on endothelial cells was similar to that of VEGI alone or of VEGI and CTT combined treatment at a concentration of 40 ng/mL. No effect on the proliferation of the endothelial cells or cancer cells was observed in the group of CTT treatment (Figure 5(b)). These results suggest that recombinant protein VEGI-CTT, which is bioactive, could specifically inhibit proliferation of endothelial cells when compared with other tested cell types.

### 3.4. Inhibition of in vitro angiogenesis by chimeric protein VEGI-CTT

The antiangiogenic activity of the recombinant VEGI-CTT was examined with an in vitro angiogenesis model. In this model, when recombinant VEGI-CTT was added to the cell

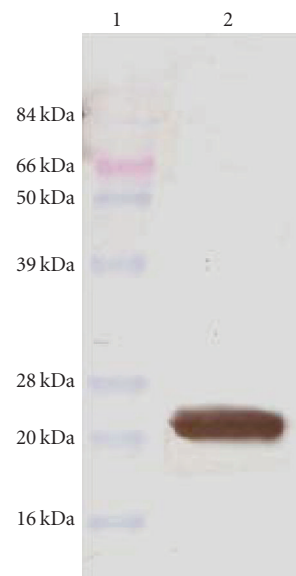


FIGURE 4: Western blotting analysis of the renatured, purified recombinant protein VEGI-CTT. Ten micrograms of VEGI-CTT were applied into 12% SDS-PAGE, and then electrotransferred to a PVDF membrane. Western blotting analysis was performed using mouse anti-His-Tag monoclonal antibody (Lane 2). The membrane was incubated with the second antibody: HRP-labeled goat antimouse IgG. Lane 1: prestained molecular weight protein markers. Lane 2: fusion protein VEGI-CTT of 22 kDa.

cultures together with FGF-2, inhibition of the formation of capillary-like tubes by ABAE cells was observed (Figure 6). The relative intensities of the tubules were determined by using computer-assisted image analysis. The  $IC_{50}$  value for the inhibition was found to be approximately 40 ng/mL. VEGI used alone or VEGI and CTT combined treatment also could inhibit the formation of capillary-like tubes by ABAE cells similar to those of VEGI-CTT, whereas the antiangiogenic activity of CTT was not observed (Figure 6). These results further demonstrated that recombinant protein VEGI-CTT had a potent activity of antiangiogenesis through inhibiting the proliferation of endothelial cells.

### 3.5. VEGI-CTT potently reduced the activity of MMP-2 and MMP-9

CTT is a recently described cyclic peptide collagenase inhibitor of MMP-2 and MMP-9. To determine whether the recombinant protein we had expressed and renatured also could efficiently inhibit the activity of MMP-2 and MMP-9, metalloproteinase inhibitory activity of VEGI-CTT was assessed by casein degradation assay. In order to precisely compare the bioactivity between recombinant protein VEGI-CTT and CTT peptide, molar concentration was used here. As shown in Figure 7, degradation of  $\beta$ -casein by MMP-9 or MMP-2 was inhibited by VEGI-CTT, similar to that of CTT or both CTT and VEGI. Moreover, the inhibitory effect of VEGI-CTT occurred in a dose-dependent manner. For MMP-2, the fusion protein VEGI-CTT inhibited MMP-

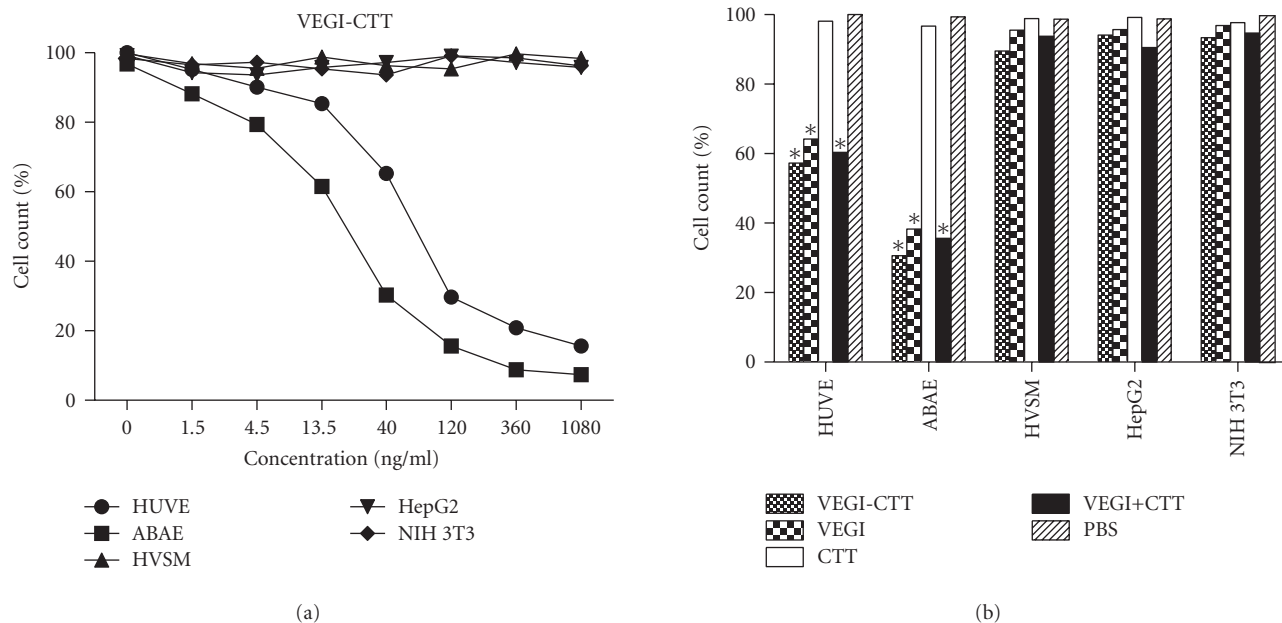


FIGURE 5: Specific inhibition of endothelial cell proliferation by chimeric protein VEGI-CTT. Cells were seeded in triplicate at 3000–5000 cells/well in 96-well plates. Culture conditions are described in Section 2. The media were changed once on day 3. The number of viable cells was determined on day 6 by using a Coulter counter. Mean values are presented as a function of VEGI-CTT or other agents' concentrations. \* $P < .05$  versus that of PBS group.

2 with an  $IC_{50}$  of 150 ng/mL (equal to  $7.5 \mu\text{M}$ ). Caseinolysis by MMP-9 was similarly inhibited by the protein at high concentrations with an  $IC_{50}$  of 650 ng/mL (equal to  $30 \mu\text{M}$ ). The differences of  $IC_{50}$  for MMP-2 and MMP-9 may be due to the different avidity of VEGI-CTT with them. However, VEGI alone could not inhibit the degradation of  $\beta$ -casein by MMP-9 or MMP-2. The data indicated that recombinant protein VEGI-CTT had similar activity to the CTT peptide as previously reported, and could potentially reduce the activity of MMP-2 and MMP-9 in a dose-dependent manner.

### 3.6. Xenograft antitumor activity

We extended our analysis of the antitumor activity of chimeric protein VEGI-CTT using mouse xenograft models. Cultured CA46 lymphoma cells were implanted subcutaneously into nude mice, the agent treatment started upon tumor xenograft development, and the tumor growth was monitored for 2 weeks. Subcutaneous inoculation of  $1 \times 10^7$  CA46 cells resulted in aggressively growing tumors in PBS treatment group (Figure 8). Tumor-bearing mice were treated with predetermined doses of chimeric protein VEGI-CTT, and significant reduction in tumor burden was observed. Treatment with 50  $\mu\text{g}/\text{mouse}$  VEGI-CTT twice a week reduced tumor growth by 82.2% compared with animals in the PBS-treated group. In contrast, treatment with 50  $\mu\text{g}/\text{mouse}$  VEGI or CTT peptide alone merely resulted in 49.2% and 35.1% reduction, respectively. In addition, compared with VEGI-CTT treatment, combined treatment with 50  $\mu\text{g}/\text{mouse}$  VEGI and CTT peptide had a relatively low antitumor activity but also significantly reduced

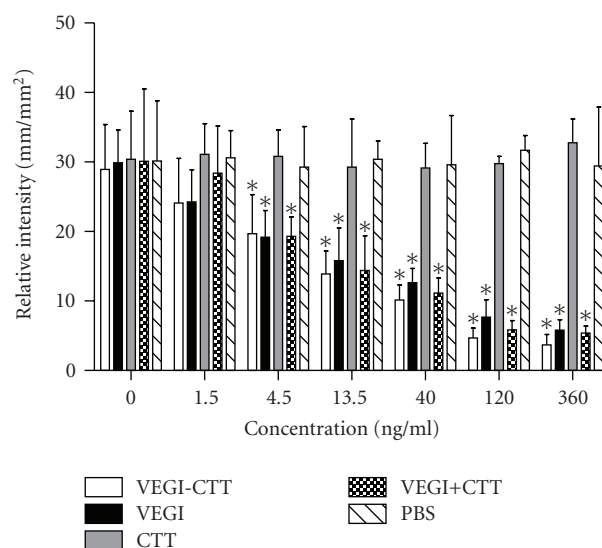


FIGURE 6: Inhibition of formation of capillary-like tubules by adult bovine aortic endothelial (ABAE) cells in collagen gels. The experiments were carried out in triplicate. At least 9 image areas were analyzed for each VEGI concentration. Mean values and standard deviations are presented as a function of VEGI concentration. \* $P < .05$  versus that of PBS group.

tumor growth by 62.9%. These results establish chimeric protein VEGI-CTT as an effective inhibitor of lymphoma tumor growth in vivo, and thus it had a more potent antitumor activity than VEGI, CTT, or the combination of both.

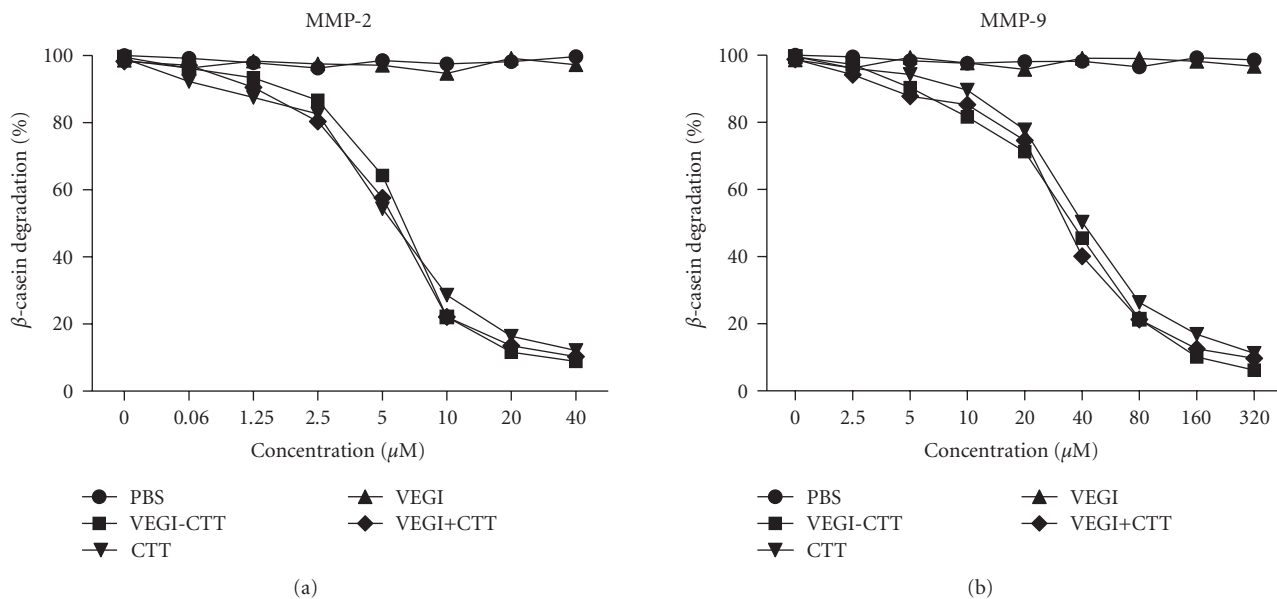


FIGURE 7: Inhibition of caseinolytic activity by the chimeric protein VEGI-CTT. MMP-2 and MMP-9 enzymes were incubated with 2 mg/mL  $\beta$ -casein in the absence or presence of VEGI-CTT. Proteins were separated on a 10% SDS-polyacrylamide gel, and detected with Serve blue R. For details, see Section 2. Inhibition of MMP-2 and MMP-9 mediated  $\beta$ -casein degradation by VEGI-CTT at different concentrations with apparent  $\text{IC}_{50}$  of  $7.5 \mu\text{M}$  and  $30 \mu\text{M}$ , respectively.

#### 4. DISCUSSION

Angiogenesis is required for tumor growth and metastasis, and its inhibition may be a new valuable approach to cancer therapy. It has been demonstrated that a variety of endogenous angiogenesis inhibitors such as angiostatin [16], endostatin [17], restin [18, 19], canstatin [20, 21], and tumstatin [22, 23] can lead to the suppression of primary and metastatic tumors' growth without affecting the normal vasculature growth. Advantages of antiangiogenic therapy include easy access of drugs to the endothelial cell compartment and low drug resistance. Vascular endothelial cell growth inhibitor (VEGI), a novel cytokine which belongs to the TNF superfamily, is one of the most potent inhibitors of endothelial cell proliferation and migration as well as tumor growth in mice [4–6]. The synthetic CTTHWGFTLC-deca peptide (CTT peptide) has been recently described as a specific and selective inhibitor for MMP-2 and -9. CTT peptide has been shown to suppress invasion and migration of both tumor and endothelial cells in vitro, prevent the growth and invasion of tumors in mice, and specifically home to tumor vasculature expressing gelatinases (MMP-2 and -9) in vivo [13, 14].

In the present study, we incorporated the extracellular domain of VEGI with CTTHWGFTLC-deca peptide for anti-tumor therapy by more potent antiangiogenesis. The fusion protein VEGI-CTT was expressed in *E. coli*. Recombinant proteins expressed in bacteria often form inclusion bodies, especially when they are expressed at high levels [24]. In our case, the expressed protein formed inclusion bodies. Therefore, an efficient and convenient refolding system that would help the purification of VEGI-CTT is needed [25]. Renaturation of VEGI-CTT is very crucial because

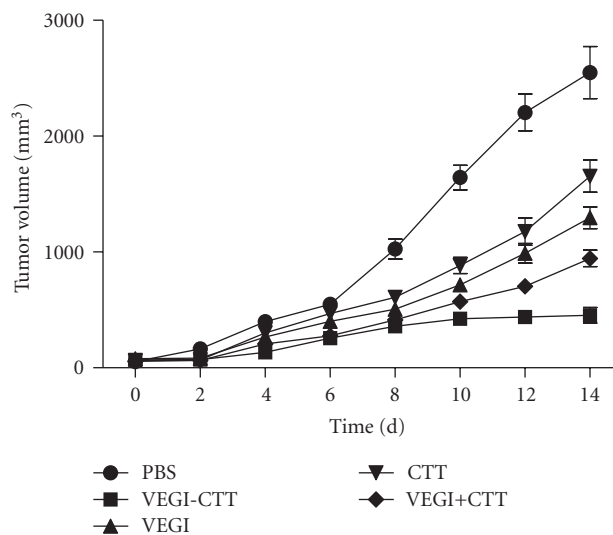


FIGURE 8: Effect of chimeric protein VEGI-CTT in treating CA46 human lymphoma xenograft-bearing nude mice. Nude mice were inoculated with CA46 human lymphoma cells, and the treatment started upon tumor xenograft establishment in nude mice. Tumor xenograft-bearing nude mice were injected with indicated doses of VEGI-CTT, VEGI, CTT, or VEGI and CTT combined treatment for two weeks, and tumor volumes were measured on alternate days. Mean values and standard deviations are presented as tumor volumes in each group ( $n = 9$ ). The experiments were repeated with similar results.

it is closely related with the final yields. There are many methods for refolding, such as dilution, dialysis, and gel filtration [26–28]. In this work, we chose on-column metal

ion-affinity chromatography for protein refolding. The purity of inclusion bodies is a key factor. Previous reports demonstrate that the washing step can remove contaminants, especially proteins that may have been absorbed onto the hydrophobic inclusion bodies during processing and could affect protein-refolding yield [29]. In the present study, inclusion bodies were washed three times. Washing with 2 M urea using 1% Triton X-100 effectively removed remaining cellular proteins. The final purity of the inclusion bodies could reach 80% after the last washing, facilitating the following purification and refolding. Another important factor to influence the yield of refolding is the time and flow rate. We tested three different times (1, 2, and 4 hours) and three different flow rates (2, 1, and 0.5 mL/min) for on-column refolding. The result showed that there was a lot of precipitation at 2 mL/min (1, 2, and 4 hours), with some at 1 mL/min (1 and 2 hours) and 0.5 mL/min (1 hour) and little at 0.5 mL/min (4 hours). We chose 0.5 mL/min (4 hours) as the final flow rate for refolding process. This provided the opportunity to generate more soluble refolded VEGI-CTT and increase the yield of the protein. In our study, we use Ni<sup>2+</sup>-chelating column to refold, purify, and reform disulfide bonds of VEGI-CTT in one step. By this strategy, an amount of the obtained refolded recombinant VEGI-CTT was approximately 25.5 mg/L of initial bacterial broth. The results indicate that this process for renaturation and purification of VEGI-CTT is suitable and effective.

Our purpose was to express and purify VEGI-CTT, and use active VEGI-CTT to obtain more potent antitumor effect than that of VEGI or CTT peptide alone. So we confirmed the bioactivities of VEGI-CTT by a series of assays in vitro. The results showed that VEGI-CTT has both activities exerted by VEGI or CTT alone. VEGI-CTT could effectively inhibit endothelial cell proliferation and the formation of capillary-like tubes by ABAE cells; meanwhile, VEGI-CTT could potentially reduce the activity of MMP-2 and MMP-9. Importantly, the further in vivo study demonstrated that chimeric protein VEGI-CTT has more potent antitumor activity not only than that of VEGI or CTT, but also than that of the addition of VEGI and CTT in combination. The explanation may be that chimeric protein VEGI-CTT can more efficiently increase the half-life of CTT than CTT peptide used alone in vivo. Further studies with recombinant VEGI-CTT are underway to test the ability to inhibit tumor invasion or metastasis in vivo, which may give insight into its unique molecular mechanisms underlying the antitumor actions of VEGI-CTT.

## ABBREVIATIONS

VEGI: Vascular endothelial cell growth inhibitor  
 MMP: Matrix metalloproteinase  
 MMP-2: 72-kDa gelatinase/type IV collagenase  
 MMP-9: 92-kDa gelatinase/type IV collagenase.

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