# Endostatin inhibits angiogenesis by suppressing matrix metalloproteinase-2

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**Abstract: AIM** To study mechanisms involved in antiangiogenic effect of endostatin. **METHODS** Human umbilical vein endothelial cells (HUVEC) were isolated and treated with basic fibroblast growth factor (bFGF). 3D-collagen gel model was used to determine the angiogenesis of HUVEC. Activity of matrix metalloproteinase (MMP) in culture medium was analyzed by gelatin zymography and Western blot. RT-PCR was used to determine mRNA levels of MMP. **RESULTS** Endostatin inhibited the angiogenesis of HUVEC induced by bFGF. Endostatin inhibited the expression and the mRNA level of MMP-2 in a concentration-dependent manner. **CONCLUSION** MMP-2 inhibition plays an important role in antiangiogenic effect of endostatin.

**Key words**: endostatin; fibroblast growth factor, basic; umbilical veins; matrix metalloproteinase

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Angiogenesis is one of the important steps involved in tumor growth and metastasis[1]. Consequently, medications to inhibit the angiogenesis provide a promising opportunity to overcome tumor. A number of endogenous antiangiogenic molecules have been identified. These include angiostatin, endostatin, antithrombin fragment and some other small molecules[2,3]. Endostatin, a $M_r$ 20 000 protein, can specifically and strongly inhibit tumor angiogenesis. The endostatin gene has been cloned and expressed as a recombinant protein in *Escherichia coli* and yeast expression system[2,4]. Systemic administration of recombinant endostatin caused growth regression of a variety of murine and xenotransplanted human tumors[5]. Many mechanisms of antiangiogenic effect about endostatin had been reported. Endostatin inhibits angiogenesis at the cellular level by inhibiting the endothelial cell cycle, by increasing endothelial cell apoptosis and by impairing vessel maturation[6]. But, the molecular targets of endostatin are not clearly understood.

*In vitro* studies of angiogenesis have been limited by the lack of a simple model of capillary wall. In this study, human umbilical vein endothelial cells (HUVEC) were cultured on the surface of a three-dimensional gel of type Ⅰ collagen, a major constituent of the pericapillary connective tissue, with the purpose of determining what molecular targets are implicated in the antiangiogenic effect of endostatin.

## 1 MATERIALS AND METHODS

### 1.1 Drug and reagents

Recombinant human endostatin (purity ⩾ 98%) was a gift from Prof. LUO Yong-Zhang, Tsinghua University. Medium 199 (M199) was purchased from Gibco/BRL. Endothelial cell growth supplement and basic fibroblast growth factor (bFGF) were obtained from Sigma Co..

### 1.2 Human umbilical vein endothelial cells culture

HUVEC were isolated from human umbilical veins by collagenase treatment as described previously[7]. Cells were cultured in M199 supplement-

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ed with 20% fetal bovine serum (FBS), 100 kU·$L^{-1}$ penicillin, 100 mg·$L^{-1}$ streptomycin, 20 mg·$L^{-1}$ endothelial cell growth supplement, and 90 mg·$L^{-1}$ heparin at 37℃ under a humidified mixture of 95% air and 5% $CO_2$. The endothelial nature of these cells was ascertained by the following criteria: presence of factor Ⅷ-related antigen, as determined by immunohistochemistry; and typical "cobblestone" appearance of confluent cultures. Contamination by smooth muscle cells or fibroblasts was not observed. HUVEC in passages 2 – 6 were used in the following assays.

### 1.3 Preparation of 3D-collagen gels

Rat tail tendons (3 g) were extracted with 100 mL sterile 1∶1000 (*V*/*V*) acetic acid solution for 48 h at 4℃. The resulted solution was centrifuged at 16 000 × *g* at 4℃ for 1 h to remove undissolved debris. The supernatant was then extensively dialyzed against distilled water and stored at 4℃.

Gels of reconstituted collagen fibers were prepared by simultaneously raising the pH and ionic strength of the collagen solution. This was achieved by quickly mixing 7 volume of cold collagen solution with 1 volume of 10 × M199 and 2 volume of sodium bicarbonate (11.76 g·$L^{-1}$) in a sterile flask. About 1 mL mixture was then dispensed into a 35 mm plastic culture dish (Corning Costar, Cambridge, MA, USA) and allowed to gel for 10 min at 37℃[9].

### 1.4 Endothelial tube formation assay

HUVEC were trypsinized and seeded into 35 mm 3D-collagen gel-coated dishes and were grown to confluence. Then, bFGF (5 μg·$L^{-1}$) with or without indicated concentrations of endostatin were added into each dishes. On the d 2 – d 10, the number of the tubes below the gel surface was determined using phase-contrast microscope at 10 randomly selected × 200 magnification fields. The culture medium was collected for gelatin zymography.

### 1.5 Gelatin zymography

Gelatinolytic activities of secreted matrix metalloproteinase (MMP) in the culture medium were analyzed by zymography on gelatin-containing polyacrylamide gels. Samples were prepared in nonreducing loading buffer. The conditioned media were separated by SDS-PAGE using 10% acrylamide copolymerized with 0.1% gelatin. After electrophoresis, the gel was washed twice for 15 min in washing buffer (50 mmol·$L^{-1}$ Tris-HCl, pH 7.5, 150 mmol·$L^{-1}$ NaCl, 10 mmol·$L^{-1}$ $CaCl_2$, 1 μmol·$L^{-1}$ $ZnCl_2$, and 0.1% Triton X-100) to remove SDS and then incubated at 37℃ for 24 h in incubation buffer (50 mmol·$L^{-1}$ Tris-HCl, pH 7.5, 150 mmol·$L^{-1}$ NaCl, 10 mmol·$L^{-1}$ $CaCl_2$, and 1 μmol·$L^{-1}$ $ZnCl_2$). Gels were stained with 0.25% coomassie brilliant blue R-250.

### 1.6 Western blot analysis

The presence of MMP-2 was confirmed by Western blot using polyclonal antibodies. Conditioned medium was concentrated and subjected to SDS/PAGE under reducing conditions, using 10% polyacrylamide gels. Proteins were transferred electrophoretically to 0.45 μm nitrocellulose membranes. The primary antibodies were used at a concentration of 10 mg·$L^{-1}$. The secondary antibodies were horseradish peroxidase conjugated goat anti-mouse and were used at a 1∶1000 dilution.

### 1.7 Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cultured cells using phenol and guanidinium thiocyanate (Tri-reagent, Gibco). Synthesis of cDNA was carried out with reverse transcriptase (M-MLV, Gibco): 500 ng of total RNA was reverse-transcribed into single-stranded cDNA using the buffer of 50 mmol·$L^{-1}$ Tris-HCl (pH 8.3), 75 mmol·$L^{-1}$ KCl, 3 mmol·$L^{-1}$ $MgCl_2$, 10 mmol·$L^{-1}$ DTT, 0.5 μg oligo-dT and 0.5 mmol·$L^{-1}$ dNTPs (dNTP Mix, Gibco), incubated at 37℃ for 1 h. After inactivation at 95℃ for 5 min, 100 ng of cDNA product was added to a 50-μL PCR reaction mixture consisting 50 pmol corresponding primers of the indexes to be detected and β-actin; 0.2 mmol·$L^{-1}$ dNTPs; 10 mmol·$L^{-1}$ Tris-HCl (pH 8.3), 50 mmol·$L^{-1}$ KCl, 1.5 mmol·$L^{-1}$ $MgCl_2$ and 0.5 μL Taq DNA polymerase (Promega, USA). As negative control, the DNA template was omitted in the reaction. β-actin was amplified as an internal con-

trol for quantitation under the same PCR conditions. PCR products were electrophoresed on 1.5% agarose gels.

### 1.8 Statistical analysis

Data were expressed as $x \pm s$ and analyzed using $t$ test. $P < 0.05$ was considered statistically significant.

## 2 RESULTS

### 2.1 Effect of bFGF on human umbilical vein endothelial cells in 3D-gel

Cells grown to confluence on the 3D-collagen gels remained on the gel surface and presented a typical cobblestone-like monolayer. After treating the cells with bFGF (5 $\mu g \cdot L^{-1}$) for 24 h, cells took on an irregular shape with elongated cell morphology. Examination under phase contrast microscope clearly showed that these cells began to infiltrate into the collagen matrix. About 2 – 4 d after incubation with bFGF, the invading cells organized into short-cord structures under the gel surface. While, control cells in normal medium were confined to the surface (Fig 1).

### 2.2 Endostatin inhibiting angiogenesis

To test the effect of endostatin on the bFGF-induced angiogenesis *in vitro*, tube formation assay was performed in the 3D-gel model. Endostatin reduced the tube structures under the cell monolayer in a concentration-dependent manner. And on the d 10, the inhibition percentages of endostatin (20 and 40 $mg \cdot L^{-1}$) were 24.2% and 33.3%, respectively (Fig 2).

### 2.3 Effect of endostatin on matrix metalloproteinases

It has been reported that MMP plays an

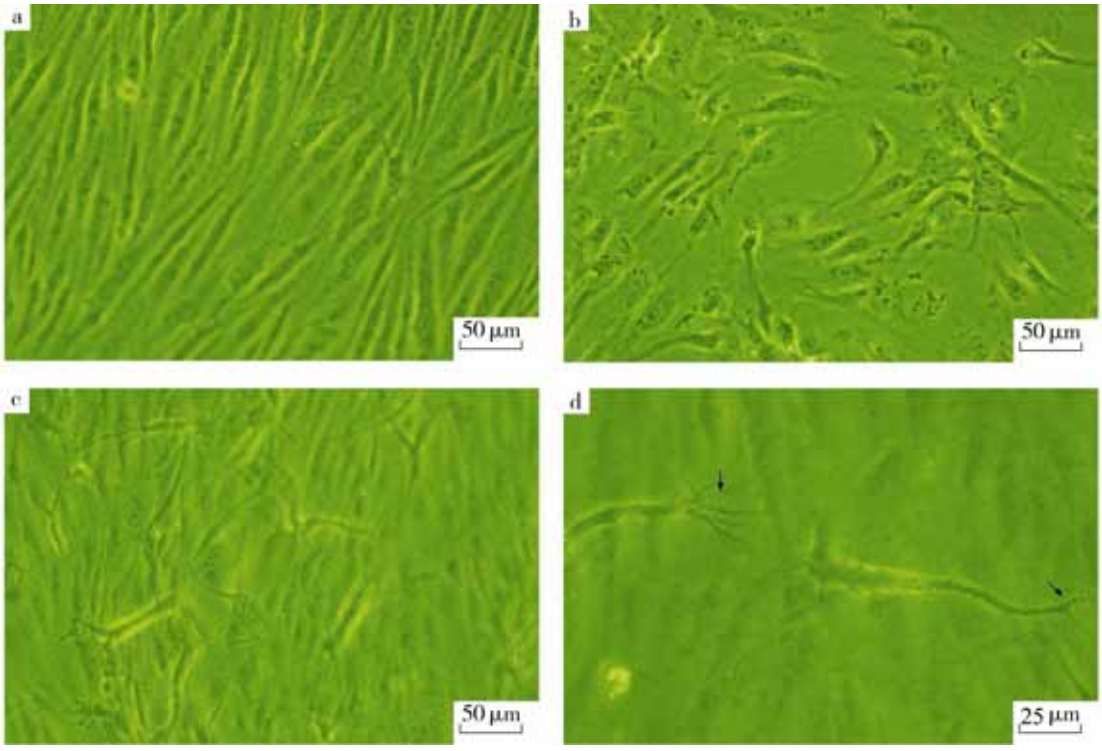


**Fig 1. Effect of basic fibroblast growth factor (bFGF) on human umbilical vein endothelial cells (HUVEC), grown on 3D-gel matrix (phase-contrast microscopy).** (a) Control HUVEC form a monolayer on gel surface. (b) HUVEC treated with bFGF 5 $\mu g \cdot L^{-1}$ for 24 h showed an irregular shape, and began to infiltrate. (c) 2 – 4 d after incubation with bFGF, HUVEC organized into short capillary-like structures under the gel surface. (d) Endothelial cells with filopodia extending into the gel matrix after bFGF incubation for 8 – 10 d (arrows).

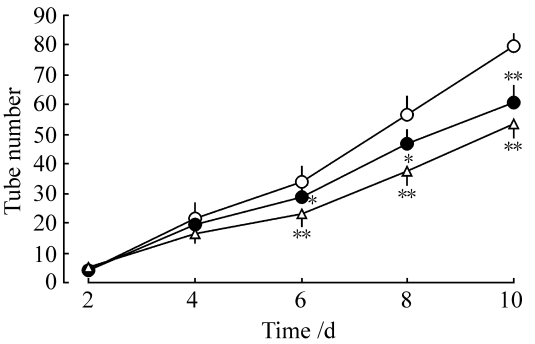


**Fig 2. Effect of endostatin on angiogenesis of HUVEC.** All groups received bFGF (5 $\mu g \cdot L^{-1}$) and endostatin for 10 d, simultaneously. (○, ●, △) endostatin 0, 20 and 40 $mg \cdot L^{-1}$. $\bar{x} \pm s$, $n = 10$. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control (0 $mg \cdot L^{-1}$).

important role in angiogenesis and tumor development[10]. Does endostatin inhibit invasion and tube formation through the metalloproteinases involved signaling pathway? To investigate this question, the culture medium on d 10 was subjected to gelatin zymography, which was able to detect the activity of secreted MMPs. As shown in Fig 3, the main bands at 64 and 72 ku corresponded respectively to active and latent MMP-2[11] and other MMPs activities were barely detected in the assay. After treatment with bFGF, the MMP-2 activity was increased (Fig 3, lane 3), while treating the HUVEC with bFGF and endostatin or MMP inhibitor, batimastat, resulted in the suppressed activity of both active and latent MMP-2 (Fig 3, lanes 4 – 6).

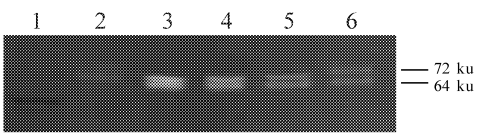


**Fig 3. Effect of endostatin on matrix metalloproteinases.** Cultured medium on d 10 was subjected to gelatin zymography. Lane 1, marker; lane 2, no treatment; lane 3, treatment with 5 $\mu g \cdot L^{-1}$ bFGF alone; lanes 4 and 5, treatment with bFGF and endostatin (20 and 40 $mg \cdot L^{-1}$, respectively); lane 6, treatment with bFGF and batimastat (0.15 $\mu mol \cdot L^{-1}$). The results were obtained from a representative experiment performed in triplicate.

The inhibitory effect of endostatin on MMPs was further confirmed by gelatin zymography and Western blot. Cells were treated by bFGF and various concentrations of endostatin for 24 h. Conditioned medium were collected for the assay. Endostatin effectively inhibits the bFGF-induced MMP-2 activity in a dose-dependent manner (Fig 4).

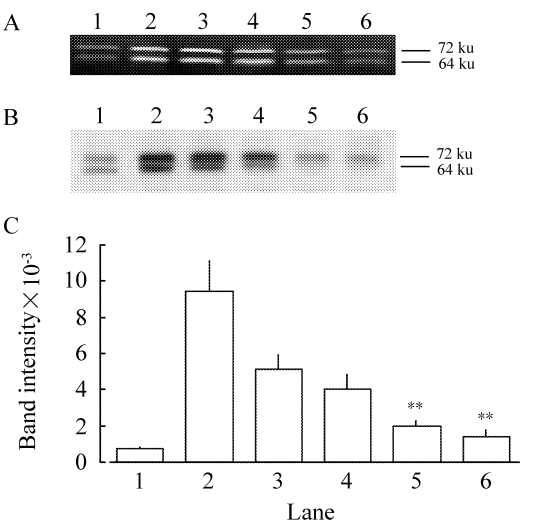


**Fig 4. Gelatin zymography (A) and Western blot (B) analysis of MMP-2 in the conditioned medium of HUVEC.** Conditioned medium of HUVEC was generated for 24 h in the absence or presence of bFGF and various concentrations of endostatin. Lane 1, no treatment; lane 2, treatment with 5 $\mu g \cdot L^{-1}$ bFGF alone; lanes 3 – 6, treatment with bFGF and endostatin (5, 10, 20 and 40 $mg \cdot L^{-1}$, respectively). The results were obtained from a representative experiment performed in triplicate. Band intensities of Western blot were examined by scanning densitometry (C). $\bar{x} \pm s$, $n = 3$. $^{**}P < 0.01$, compared with lane 2.

## 2.4 Effect of endostatin on matrix metalloproteinases mRNA levels

To evaluate expression of MMP mRNA in HUVEC treated with bFGF and endostatin, RT-PCR was performed on extracted mRNA aliquots from serum-free cultured cells. All of the primer sequences, PCR product sizes and PCR conditions are listed in Tab 1. As shown in Fig 5, the mRNA levels of MMP-2 were increased while treating the cells with bFGF (Fig 5, lane 3), and were down regulated by endostatin in a concentration-dependent manner (Fig 5, lanes 4 – 7). However, mRNA levels of MMP-3 and MMP-9 were not

**Tab 1. Nucleotide sequences of primers used for RT-PCR and related information**

| Oligonucleotide primer(5′ – 3′) | Product size/bp | PCR condition |
|---|---|---|
| β-actin (internal standard) | | |
| F:GTAGACATCCGCAAAGAC | 303 | same as co-amplified |
| R:GAAAGGGTGTAACGCAACT | | MMPs |
| MMP-2 | | |
| F:GGATGATGCCTTTGCTCG | 599 | 94℃ 50 s, 56℃ 50 s, |
| R:CAGTGGACATGGCGGTCT | | 72℃ 1 min, 30 cycles |
| MMP-3 | | |
| F:GGCAGTTTGCTCAGCCTATC | 219 | 94℃ 50 s, 53℃ 50 s, |
| R:TCCAGAGTGTCGGAGTCCAG | | 72℃ 1 min, 30 cycles |
| MMP-9 | | |
| F:GGGACGGCAATGCTGATG | 506 | 94℃ 50 s, 59℃ 50 s, |
| R:AGGGCGAGGACCATAGAGG | | 72℃ 1 min, 30 cycles |

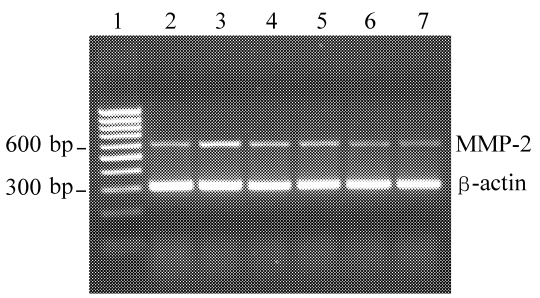


**Fig 5. Electrophoresis analysis of PCR products for MMP-2.** RNA was extracted from cultured cells treated for 24 h with bFGF or endostatin. Lane 1, marker; lane 2, control; lane 3, 5 $\mu g \cdot L^{-1}$ bFGF alone; lanes 4 – 7, bFGF and endostatin (5, 10, 20 and 40 $mg \cdot L^{-1}$, respectively). The results were obtained from a representative experiment performed in triplicate. A single band at 303 bp was internal control with primers specific for β-actin with the same cDNA under identical condition.

affected obviously (results not shown).

## 3 DISCUSSION

Although angiogenesis has been mostly studied *in vivo*, for example, in the rabbit cornea or the chorioallantoic membrane of the chicken embryo, the methods of isolation and culture of endothelial cells provided a more convenient way to study angiogenesis *in vitro*. Endothelial cells were cultured in the surface of type Ⅰ collagen gel, and were induced to infiltrate the gel matrix. This model originally described by Montesano, *et al*[11] represents a simple model of angiogenesis in which the induction or the prevention of endothelial cell invasion by exogenous signals can be easily detected.

Phorbol myristate acetate (PMA), a well-known tumor promoter, was commonly used to induce angiogenesis *in vitro*[11]. But PMA is not a physiologically occurring substance. In this study, bFGF, best-characterized angiogenic substances, were used as inducer. They are potent mitogens for several cell types, including vascular and capillary endothelia cells, and are capable of inducing an angiogenic response *in vivo*[12]. Furthermore, they are proved to be the crucial substances in the signal transduction pathway of angiogenesis[12].

An initial step in the process of angiogenesis is the degradation of matrix proteins of the basement membrane of endothelial cells. Following matrix remodeling, endothelial cells are able to migrate, proliferate and form capillary tubes[10,13]. It is generally assumed that MMP play an important role in angiogenesis. MMP-1, MMP-2 and MMP-3 are reportedly to be produced by endothelial cells, and MMP-2 is the most important one among them[10]. Data of this study showed that inhibition of the MMP resulted in the suppressed angiogenesis. This was consistent with the possible role of MMP in the angiogenic process. Endostatin treatment significantly inhibited the MMP activity in the culture medium. Furthermore, the MMP-2 mRNA level was down regulated by endostatin treatment, although MMP-1 and MMP-3 mRNA levels were not influenced. Therefore, we suggest that endostatin inhibit the activity of MMP-2 to exert its antiangiogenic effect.

However, angiogenesis is a complex process involving a series of sequential steps[14], including the degradation of the basement membrane, migration of endothelial cells towards angiogenic stimuli, proliferation of endothelial cells, formation of capillary lumina and maturation of blood vessels. Endostatin could inhibit any one or more of these steps to exert its antiangiogenic effects. And where and how does endostatin interfere in

the metalloproteinases involved signaling pathway are not fully understood in the present study and are under investigation.

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# 内皮抑制素通过抑制基质金属蛋白酶-2 而抑制血管新生


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**摘要: 目的**　研究内皮抑制素抑制血管新生作用的机制。**方法**　用碱性成纤维细胞生长因子(bFGF)处理体外原代培养人脐静脉内皮细胞;用三维胶原模型研究 bFGF 诱导的血管新生作用;用明胶电泳和蛋白印迹法研究分泌于培养基中的基质金属蛋白酶的活性和含量;RT-PCR 法研究基质金属蛋白酶的 mRNA 水平。**结果**　内皮抑制素显著抑制 bFGF 诱导的内皮细胞血管新生能力;抑制内皮细胞分泌的基质金属蛋白酶-2 的表达及其 mRNA 的水平。**结论**　内皮抑制素通过抑制基质金属蛋白酶-2 的表达而抑制血管新生。



**关键词:** 内皮抑制素; 成纤维细胞生长因子, 碱性; 脐静脉; 基质金属蛋白酶

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