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Endostar, a novel recombinant human endostatin, exerts antiangiogenic effect via blocking VEGF-induced tyrosine phosphorylation of KDR/Flk-1 of endothelial cells

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Abstract

Endostar, a novel recombinant human endostatin expressed and purified in *Escherichia coli* with an additional nine-amino acid sequence and forming another his-tag structure, was approved by the SFDA in 2005 for the treatment of non-small-cell lung cancer. But its mechanism of action has not been illustrated before. In this study, we examined the antiangiogenic activities of endostar *in vitro* and *in vivo*. The results showed that endostar suppressed the VEGF-stimulated proliferation, migration, and tube formation of human umbilical vein endothelial cells (HUVECs) *in vitro*. Endostar blocked microvessel sprouting from rat aortic rings *in vitro*. Moreover, it could inhibit the formation of new capillaries from pre-existing vessels in the chicken chorioallantoic membrane (CAM) assay and affect the growth of vessels in tumor. We further found the antiangiogenic effects of endostar were correlated with the VEGF-triggered signaling. Endostar suppressed the VEGF-induced tyrosine phosphorylation of KDR/Flk-1(VEGFR-2) as well as the overall VEGFR-2 expression and the activation of ERK, p38 MAPK, and AKT in HUVECs. Collectively, these data indicated the relationship between endostar and VEGF signal pathways and provided a molecular basis for the antiangiogenic effects of endostar.

Keywords: Endostar; Angiogenesis; HUVEC; VEGF; KDR/Flk-1

The angiogenesis inhibitors for the treatment of cancer as a new approach are based on the Forkman's theory in 1971 [1]. From then on, between more than three decades of research on angiogenesis, hundreds of angiogenesis inhibitors was discovered in the lab and used in the drug development. Until the 2004, avastin was firstly approved by the FDA may be a hallmark of new anti-tumor times. But there are significant problems with the anti-VEGF monoclonal antibodies. For example mutant tumor cells may over time produce redundant angiogenic factors, which affect the long-term use of the drug especially in the prognosis of tumor [2]. Therefore, it was necessary to develop broad-spectrum and low-toxicity agents. Actually there were some prospective ones been discovered and developed from then on including endostatin.

Endostatin, the 20 kD internal fragment of the carboxy-terminus of collagen XVIII, was first identified in the conditioned media of hemangioendothelioma cells as an antiangiogenic molecule in 1997 by Folkman et al. in his laboratory at the Children's Hospital in Boston [3]. Animal studies demonstrated that endostatin strongly inhibited the growth of a variety of murine and xenotransplanted human tumors by suppressing the neovascularization [4,5]. On the cellular level, it was reported that endostatin inhibits endothelial cell proliferation [3] and migration [6,7] and induces endothelial cell apoptosis [8,9] and cell cycle arrest [10]. Endostatin thought to be an ideal anticancer weapon,

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was quickly pushed into clinical trials [11–13]. However, the anti-tumor effect of recombinant human endostatin was not satisfied in the clinic trials. It was apparently lack of efficacy because of the problem in recombinant technique [14,15]. Thus far, many efforts have been focused on it. The recombinant endostatin prepared from *Escherichia coli* is insoluble after purification and therefore inappropriate for clinical settings. A soluble form of endostatin is available from a yeast system that has a relatively low yield and high cost, which has made it difficult to produce endostatin in quantities sufficient for extensive clinical evaluation [16].

Endostar, a novel recombinant human endostatin which expressed and purified in *E. coli*, was approved by the SFDA for the treatment of non-small-cell lung cancer in 2005. Compared with rh-endostatin reported in previous literature, an additional nine-amino acid sequence (MGGSHHHHH) was added at the N-terminal of the protein, which resulted in the formation of a six-histidine tag. The six-histidine tag could be chelated with metal ions such as Ni^{2+} with a relatively high affinity. These changes simplify purification and improve the stability of protein [17]. But whether the changes of the structure affects the antiangiogenic efficacy including the mechanism of action remain unknown.

In this study, we assessed the antiangiogenic efficacy of endostar *in vitro* and *in vivo*, and also investigated its mechanism of action on VEGF signal transduction.

Materials and methods

Material. Endostar, expressed and purified in *E. coli*, was provided by Simcere Pharmaceutical Research Co., Ltd. Recombinant human VEGF was purchased from Chemicon International. Primary antibodies for p38, p-p38, Akt, p-ERK1/2, PECAM-1, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibody for ERK1/2 was from Chemicon. Antibodies for p-VEGFR2 and VEGFR2 were from Cell Signaling Technology (Beverly, MA). IRDyeTM800 conjugated secondary antibodies were obtained from Rockland Inc.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by collagenase treatment as described previously [18]. The harvested cells were grown in medium 199 (Gibco, Grand Island, NY, USA) containing 20% heat-inactivated fetal calf serum (Gibco), endothelial cell growth supplement (ECGS, 30 μg/ml, Sigma), epidermal growth factor (EGF 10 ng/ml, Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin. After 3–5 passages, HUVECs were collected for use in all experiments.

Rat aortic ring assay. Rat aortic ring assay was performed as described previously with some modification [19]. Clotting media contained $M199^+$ (M199 with 100 U/ml penicillin and 100 μg/ml streptomycin) plus 0.3% fibrinogen and 0.5% ε-amino-*n*-caproic acid (ACA; Sigma, St. Louis, MO). Growth media consisted of $M199^+$ with 20% fetal bovine serum and 0.5% ACA. Endostar was reconstituted in $M199^+$ and then added to the growth media in the appropriate concentrations. Plates were then stored in incubator at 37 °C and 5% CO_2. Growth media were only changed when a new treatment was added.

Endothelial cell migration assay. Chemotactic motility of HUVECs was assayed using Transwell (Millicell, Billerico, USA) with 6.5-mm diameter polycarbonate filters (8-μm pore size) as described previously [20]. Various concentrations of endostar were given to the cells for 30 min at room temperature before seeding. Hundred microliters of the cell suspension was loaded into each of the upper wells. The chamber was incubated at 37 °C for 4 h. Cells were fixed and stained with H&E. Nonmigrating cells on the upper surface of the filter were removed by wiping with a cotton swab, and chemotaxis was quantified by counting with an optical microscope (400×) the cells that migrated to the lower side of the filter. Ten fields were counted for each assay. The inhibition rate of migration was calculated using the formula: inhibition rate of migration $= [1 - (\text{the migrating cells})_{\text{Endostar}}/(\text{the migrating cells})_{\text{Control}}] \times 100\%$.

Tube formation assay. Tube formation assay was performed as described previously [21]. Various concentrations of endostar were given to HUVECs for 30 min at room temperature before seeding and plated onto the layer of matrigel at a density of 1×10^4 cells/well, and followed by

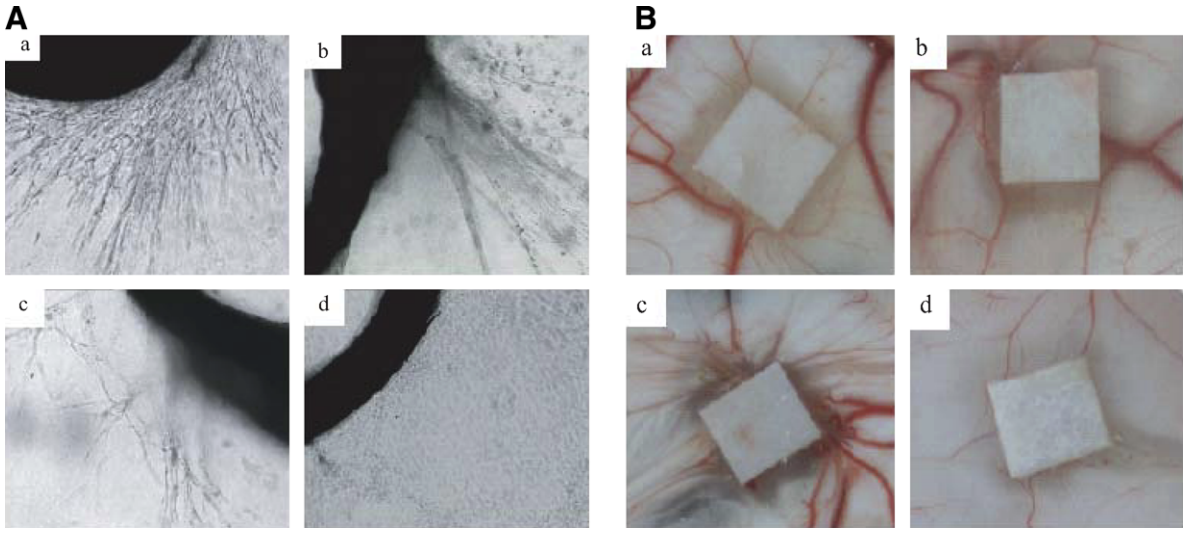

Fig. 1. (A) Endostar inhibits rat aortic ring microvessel sprouting. Photographs represent aortic rings grown for 7 days in growth media. (a) Control, PBS; (b) 80 μg/ml endostar; (c) 200 μg/ml endostar; and (d) 500 μg/ml endostar. Photographs are representative pictures from three independent experiments. (B) Endostar inhibits angiogenesis *in vivo*. After fertilized chicken eggs were incubated for 9 days, filter paper disks saturated with endostar (0.15–1.35 μg per egg) or PBS were placed on the CAMs. After a 48-h incubation, a fat emulsion was injected under the CAMs for better visualization of the vessels. Disks and surrounding CAMs were photographed. (a) Control, PBS; (b) 0.15 μg endostar per egg; (c) 0.45 μg endostar per egg; and (d) 1.35 μg endostar per egg. Photographs are representative pictures from three independent experiments.

the addition of 5 ng/ml VEGF. After 8 h, the enclosed networks of complete tubes from five randomly chosen fields were counted and photographed under a microscope. The inhibition rate was calculated using the following formula: Inhibition rate of tube formation $= [1 - (\text{tubes}_{\text{Endostar}}/\text{tubes}_{\text{Control}})] \times 100\%$.

Chicken chorioallantoic membrane (CAM) assay. Antiangiogenic activity of endostar on CAM was assayed, as described with modification [22]. Filter paper disks (5 × 5mm) saturated with or without endostar were placed on the CAMs. The eggs were then incubated at 37 °C for another 2 days, and then the CAMs were photographed. Ten eggs were tested for each group, and the assay was performed twice to ensure reproducibility.

Effect of GA on growth of inoculated tumor Lewis in mice. C57BL/6 mice with body weight of 18–22 g were subcutaneously injected with Lewis lung cancer cells in a volume of 0.2 ml (10^8/ml) [23]. After 24 h of tumor inoculation, model animals were at random divided into 4 groups, and each group contained 10 mice, half male and half female. Three groups were received different dosages of endostar treatment: 5, 10, and 50 mg/kg, respectively. The control group received 0.9% normal saline. Endostar and vehicle were given through i.v. injection 24 h after tumor inoculation at a frequency of once every other day for a total of 10 days. After the treatments, all mice were sacrificed and weighed simultaneously, and then tumor was segregated and weighed. And the assay was performed third times to ensure reproducibility.

Immunohistochemistry. Tissue sections were incubated with 0.01 M natrium citricum for antigen retrieval. Then the slides were processed using a SP-9000 Histostain™—Plus Kits (Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd.). Primary antibody was used. The tissues then were incubated with the secondary biotinylated anti-species antibody and labeled using a modification of the avidin–biotin complex immunoperoxidase staining procedure. Positive staining was visualized using the chromagen.

Western blotting analysis. HUVECs were cultured in M199 containing 1% FBS for 6 h and then incubated with various concentrations of endostar for 30 min before VEGF stimulation for 15 min. Detection was performed by the Odyssey Infrared Imaging System (LI-COR Inc., USA). All blots were stripped and reprobed with polyclonal anti-β-actin antibody to ascertain equal loading of protein.

Statistical analysis. All results shown represent the means ± SEM from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. All comparisons are made relative to untreated controls and significance of difference is indicated as $^*P < 0.05$.

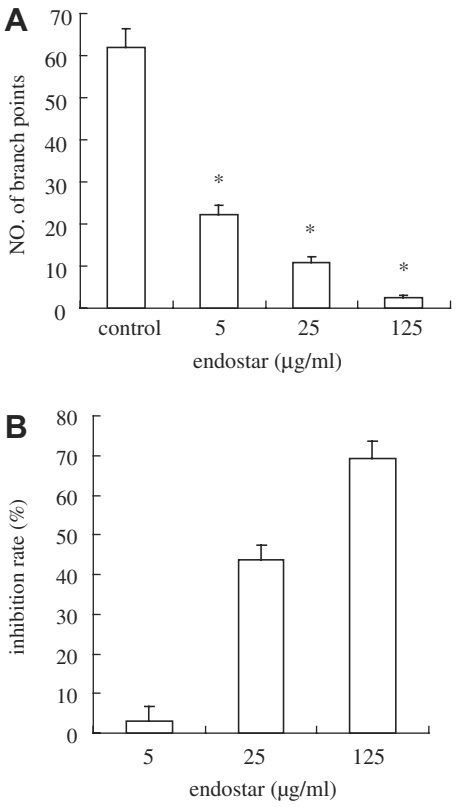

Fig. 2. (A) Effect of endostar on VEGF-stimulated HUVECs tube formation. HUVECs were pretreated for 30 min with various concentrations (5, 25, and 125 μg/ml) of endostar. Cells were collected and replated on matrigel-coated plates at a density of 1×10^5 cells/well and then incubated in the presence of 5 ng/ml VEGF. After 8 h, capillary networks were photographed and quantified. Representative endothelial tubes were shown. The quantization of the inhibition rate was represented by the number of branch point. (B) Effect of endostar on VEGF-stimulated HUVECs migration. Human umbilical vein endothelial cells (HUVECs) were pretreated for 30 min with various concentrations (5, 25, and 125 μg/ml) of endostar before treatment with 5 ng/ml VEGF. After 4-h incubation, the migrant cells were photographed and quantified as described in Materials and methods. Results are presented as the means ± SEM of data obtained in three independent experiments.

Results

Endostar inhibits angiogenesis in vitro

The Aorta Ring assay is a widely used approach of angiogenesis *in vitro* [24]. Treatment with endostar resulted in a dramatic concentration-dependent decrease in capillary sprouting; the growing sprouts were shorter and the cells that migrated into the matrix were fewer. At the tested concentrations, endostar clearly inhibited microvessel sprouting (Fig. 1A).

Endostar suppresses tube formation of human endothelial cells stimulated with VEGF

The tube formation of human endothelial cells is another method to evaluate the antiangiogenic effect of the agents [24]. When HUVECs were placed on the matrigel in the presence of VEGF (5 ng/ml), they rapidly aligned with one another and formed tube-like structures within 8 h, which required cell–matrix interaction, intercellular communication, as well as cell motility. Endostar prevented VEGF-stimulated tube formation of HUVECs in a concentration-dependent manner (Fig. 2A).

Endostar inhibits the migration of human endothelial cells stimulated with VEGF

The migration of endothelial cell is a prerequisite for angiogenesis. VEGF is a potent stimulator in this procedure [25]. After stimulating HUVECs with VEGF (10 ng/ml) for 4 h, a large number of cells migrated to the lower side of the filter in the Transwell Chamber. Five to 125 μg/ml endostar significantly reduced the number of migrated cells in a concentration-dependent manner (Fig. 2B). These results (the assay of migration and tube formation) indicated that endostar could block VEGF-induced angiogenesis *in vitro*.

Endostar can inhibits angiogenesis in vivo

As a model *in vivo* the chick embryo chorioallantoic membrane (CAM) has been utilized to study angiogenesis, antiangiogenesis effects on the chick embryo [24]. The results revealed that new blood vessels formed well on CAMs in the control group. Endostar at 0.15 μg per egg incubation for 48 h showed a notable restraint, whereas 0.45 and 1.35 μg per egg endostar drastically inhibited neovascularization of the CAM, accompanied by a lack of prominent vessel networks (Fig. 1B). These results demonstrate that endostar was able to suppress angiogenesis in embryos.

Endostar inhibited the growth of Lewis pulmonary carcinoma and reduced the tumor angiogenesis

After eight-day treatments by i.v. consecutively (5, 10, and 50 mg/kg), endostar exerted significant inhibitory effect on the growth of inoculated Lewis pulmonary carcinoma in mice. The maximum inhibition ratio at 50 mg/kg was 38.16% (Fig. 3A). Meanwhile, there was no significant difference in the average weight of endostar-treated mice compared with control mice (data not shown). Histological analysis of sections stained with an endothelial-specific antibody (PECAM-1) confirmed these macroscopic observations. The vascular density was reduced in tumors after 50 mg/kg endostar treatment (Fig. 3B).

The effects of endostar on VEGFR2 and its signaling pathways

As shown in Fig. 4, 5 ng/ml VEGF were used to induce the expression of phosphorylated VEGFR-2 in HUVECs. Pretreatment with endostar inhibited the overall VEGFR-2 expression levels and also blocked the VEGF-induced phosphorylation of KDR/Flk-1. To investigate which downstream signaling pathway was influenced by endostar, we examined the expression of ERK1/2, Akt, and p38 MAPK, and their phosphorylation status, which mainly associated with mitogenicity and proliferation. Treatment with endostar inhibited VEGF-dependent phosphorylation of ERK1/2, Akt, and p38 MAPK in a concentration-dependent manner. In all cases, the total steady state protein levels remained unchanged, suggesting that some VEGF receptors may be blocked by endostar, leading to interruption of VEGF-triggered signal.

Discussion

Some previous studies have reported that these effects of endostatin were related with vascular endothelial cell growth factor (VEGF) [7,26,27], a crucial regulator in angiogenesis. It blocks VEGF-induced tyrosine phosphorylation of KDR/Flk-1 and activation of ERK, p38 MAPK, and p125FAK, inhibits the binding of VEGF to endothelial cells and to its cell surface receptor, KDR/Flk-1[27]. In this study, we investigated the effect of endostar, a new recombinant human endostatin, on angiogenesis *in vitro* and *in vivo*, and analyzed the phosphorylation status of KDR/Flk-1 and several primary intracellular molecules in their downstream signals. The results showed that endostar definitely inhibit the activation of VEGF-triggered signal by decreasing the expression of the phosphorylation of VEGFR-2 in HUVEC. The three downstream signals have been affected by pretreatment with endostar, suggesting that endostar regulated cell survival and mitogenicity in endothelial cells by affecting pathways. Akt/PKB regulates cell survival, p44/42 MAPK controls

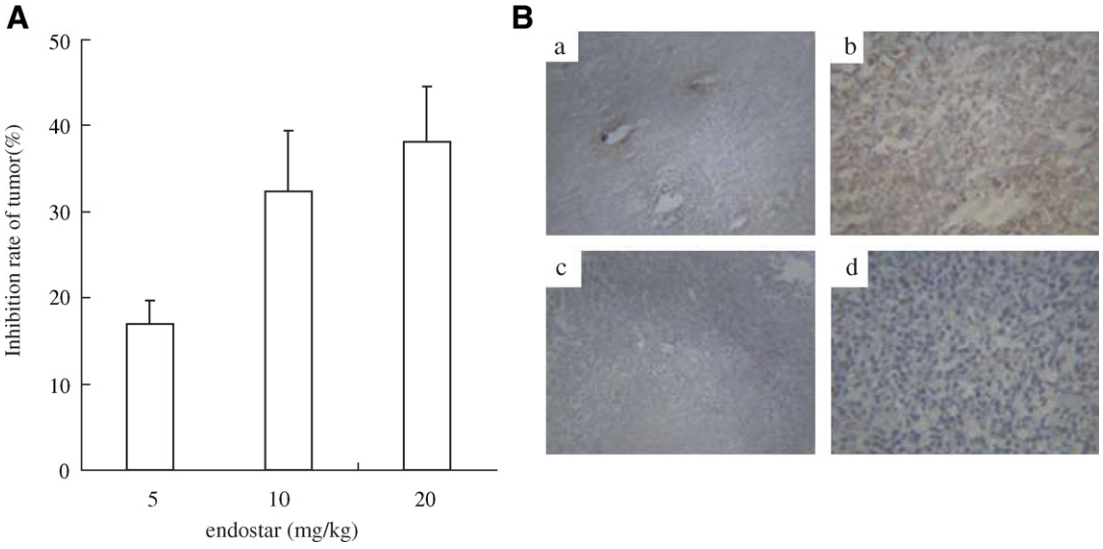

Fig. 3. (A) The anti-angiogenesis of endostar in C57 mice Lewis pulmonary carcinoma model. Endostar were given eight days by the route of i.v. consecutively, the tumor were taken pictures in different groups. (B) The immunohistochemistry of specific endothelial cell antibody (PECAM-1). (a,b), control group. (c,d), mice treated i.v. with 20 mg/kg endostar. Original magnifications, a, c, 10×; b, d, 40×.

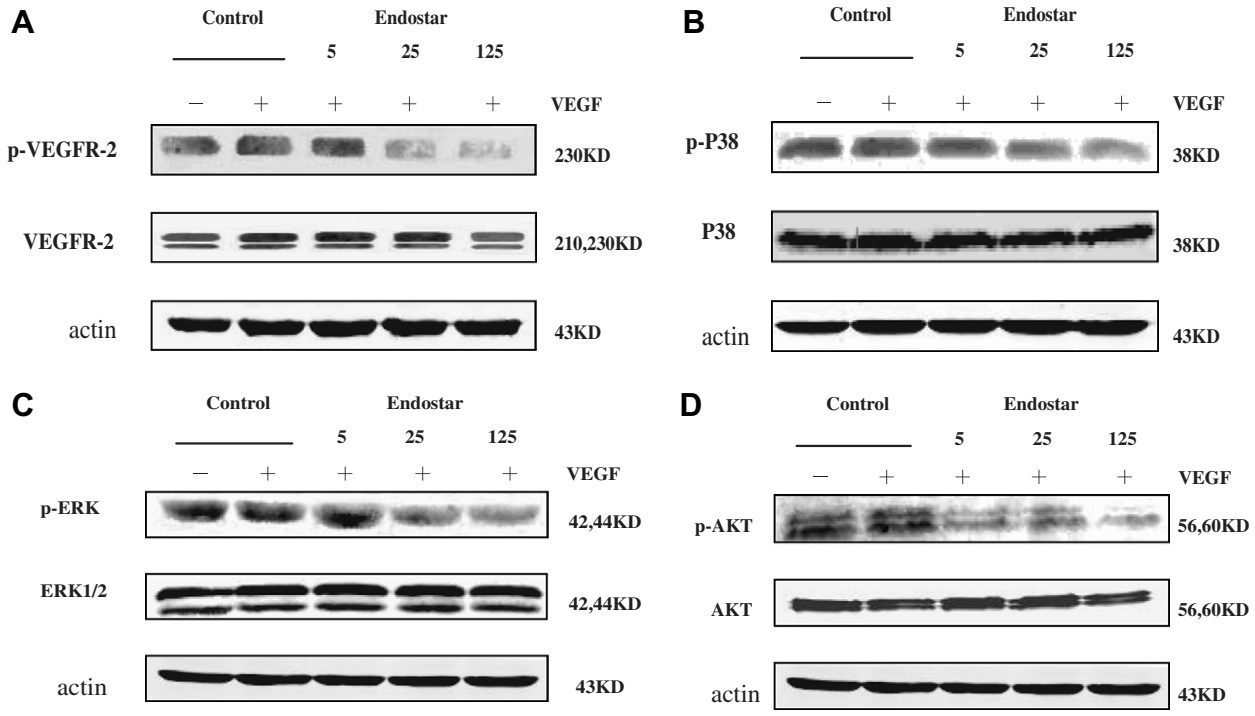

Fig. 4. The inhibition of endostar on the VEGF signal pathways. Using VEGF 5 ng/ml to induce the VEGF-triggered signal 30 min after treating with endostar 2 h. The change in quantity of the expression phosphorylation and unphosphorylated VEGFR-2 (A) and downstream signaling P38 (B), ERK1/2 (C), AKT (D) and their phosphorylation status were examined.

mitogenicity, and p38 MAPK was implicated in VEGF-mediated migration [28].

Except the above activity, is there any other molecule targeted by endostatin or endostar? In our other study, we have evaluated the effects of endostar on fibroblast growth factor (FGF)-mediated migration of primary HUVECs. The results indicated that it could inhibit the migration of HUVECs and affect vascular formation in the embryoid body model. However, we could not detect any effect of endostar on FGF-R related Ras-MAP kinase pathway, the PI_3-kinase-Akt pathway, and the PLCγ pathway. Our current study are focused on finding some other receptor tyrosine kinases interfered by endostar. Since the first report of endostatin, numerous publications focused on its broad-spectrum antiangiogenic mechanism. Endostatin appears to be dependent on binding to E-selectin as well as blocks activity of metalloproteinases 2, 9, and 13 [29] and E-cadherins [10,30,31]. The clear picture to emerge is the pervasiveness of its influence—one that may be attributable in part to a long presence of the gene over the evolutionary course of the human genome. Abdollahi et al. [32,33] reported that ~12% of all genes are significantly regulated in human microvascular endothelial cells exposed to endostatin by using custom microarrays covering ~90% of the human genome, which shed light on why the influence of endostatin is so extensive. They noted that the upregulated genes as a group include the known angiogenesis inhibitors, while the downregulated genes include the known stimulators. Revealed is a networked action of endostatin that cannot be reduced to single gene responses. To fully investigate the mechanism, it is necessary to study beyond individual molecular regulations and consider common physiological responses.

The current data revealed that endostar might exert anti-angiogenic effects via similar mechanism with endostatin. But endostar is different from endostatin because of the additional nine-amino acid sequences which improves the protein's half-life and its capacity to combine with zinc. So there might be some differences with the original one. Judah Folkman found that endostar is at least twice as potent as endostatin in animal tumor models [34]. That is to say, the function of endostatin has been improved. Does endostar have the same action profile with endostatin? Till now we have no idea. We hypothesize that endostar might also have a complex signal network, including KDR/Flk-1 signal cascade. To fully illustrate its action and mechanism, our further study will compare it with endostatin, including their antiangiogenic activity, the VEGFR binding activity, and their pharmacokinetics *in vivo*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.06.155.

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