

Original Article

Antitumor effects of Endostar on non-Hodgkin's lymphoma by regulating endothelial progenitor cells through protein kinase B-dependent pathway

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Endothelial progenitor cells (EPCs) play an important role in non-Hodgkin's lymphoma (NHL) development. Endostar is an anti-angiogenic drug designed to stop cancer by nullifying a tumor's ability to obtain oxygen and nutrients. In this study, we examined the anti-angiogenic activities of Endostar on NHL cell lines and murine xenograft model of NHL *in vitro* and *in vivo*, respectively, and explored the underlying antiangiogenic mechanism of Endostar. Results showed that Endostar may inhibit the EPC proliferation by reducing the expression of p-protein kinase B, but not p-ERK expression. Our finding could lead to a better understanding of the effects of Endostar on NHL.

Keywords endostar; non-hodgkin's lymphoma; endothelial progenitor cell; AKT; ERK

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Introduction

Endothelial progenitor cells (EPCs) mainly exist in the bone marrow after birth, and they can be mobilized to peripheral blood and homing to a target organization involved in physical or/and pathological angiogenesis in some physiological or pathological conditions [1]. Research shows that EPCs from bone marrow can participate in tumor angiogenesis and proliferation [2]. Previous studies have found that the number of EPCs is not only related to the tumor staging, and invasion degree, but also related to cancer chemotherapy sensitivity. The number of circulating EPCs can be used as the prediction of disease severity, and be a biological marker for treatment effects [3]. A recent study showed that the rate of EPCs in non-Hodgkin's lymphoma (NHL) patients was higher than that in normal person, and the rate of EPCs in aggressive NHL was higher than that in indolent NHL patients [4]. It suggested that EPCs play an important role in NHL development.

Endostar, recombinant human endostatin injection, is an antiangiogenic drug designed to stop cancer by nullifying a tumor's ability to obtain oxygen and nutrients for growth

[5]. Endostar works by inhibiting angiogenesis: the proliferous formation of new blood vessels in and around the tumor tissue. Endostar expressed and purified in *Escherichia coli* with an additional nine-amino acid sequence and a His-tag structure, was approved by State Food and Drug Administration (SFDA) of China in 2005 for the treatment of non-small-cell lung cancer [6]. But its mechanism has not been illustrated. In this study, we examined the antiangiogenic activities of Endostar on NHL cell lines and murine xenograft model of NHL *in vitro* and *in vivo*, respectively, and explored its underlying antiangiogenic mechanism.

Materials and Methods

Drugs and reagents

Endostar was a gift from Simcere Pharmaceutical (Nanjing, China). Antibodies against CD31, factor VIII-related antigen, extracellular matrix (ECM) gel, human fibronectin, and FITC-Ulex Europaeus Agglutinin-A (FITC-UEA-1) were purchased from Sigma (St Louis, USA); vascular endothelial growth factor-A (VEGF-A) were purchased from PeproTech (Rocky Hill, USA); antibodies of protein kinase B (AKT) and ERK were purchased from Cell Signaling Technology (Danvers, USA); β -actin was purchased from Santa Cruz Biotechnology (Santa Cruz, USA); PE-CD133 was purchased from eBioscience (San Diego, USA); FITC-CD34 was purchased from BD Biosciences (Franklin Lakes, USA); APC-CD309 was purchased from R&D Systems (Minneapolis, USA); Dil-ac-LDL was purchased from Molecular Probes (Leiden, The Netherlands); EGM-2MV kit was purchased from Lonza (Walkersville, USA). The Raji cell line, A20 cell line, EAhy.926, and human umbilical vein endothelial cells (HUVECs) were obtained from the China Center for Typical Culture Collection (Wuhan, China).

Isolation of mononuclear cells and cell culture

The mononuclear cells (MNCs) were fractionated from cord blood by human lymphocyte separation liquid (TBD Science, Tianjin, China). The isolated MNCs were washed

three times with EGM-2MV medium and resuspended. Cells were seeded into a 50-cm² culture bottle pre-coated with human fibronectin at 37°C, with 5% CO₂ in incubator. After 48 h of incubation, non-adherent cells and debris were aspirated, and adherent cells were washed once with complete EGM-2MV medium. Medium was changed every other day. Colonies of endothelial cells appeared in Day 7, then expanded everyday and identified as monolayers of cobblestone appearing cells. The colonies of endothelial cells were dispersed by 0.05% trypsin solution with 0.02% EDTA (Gibco, Gaithersburg, USA) and resuspended on Day 21 for the further culture. Murine B cell lymphoma cell line A20 cells, human Burkitt lymphoma cell line Raji cells, and endothelial cell line EAhy.926 cells were cultured in RPMI-1640 medium (Hyclone, Logan, USA) containing 10% fetal bovine serum human (FBS; Gibco). HUVECs were cultured in EGM-2MV medium.

MTT assay

The anti-proliferative effects of Endostar against different group of cells were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) dye uptake method as described previously [7]. Briefly, the final concentrations of Endostar were 50, 100, 200, and 400 µg/ml. Each concentration of Endostar was added to six wells, respectively. The cells in plates were treated with or without the indicated test samples for 24, 48, and 72 h. Thereafter, 20 µl MTT solution (5 mg/ml in phosphate-buffered saline) was added to each well. After incubation for 4 h at 37°C, the supernatant was removed and 150 µl dimethyl sulfoxide was added. When the blue crystals were dissolved, the optical density (OD) was detected in a microplate reader at a wavelength of 570 nm using a 96-well multiscanner autoreader (Biotech Instruments µQuant, New York, USA). The following formula was used: inhibition of cell proliferation (%) = $[1 - (\text{OD of the experimental sample} / \text{OD of the control})] \times 100\%$ ($n = 6$).

Cell tube formation

Cells were seeded into 96-well plates pre-coated with 30 µl ECM gel (10 µg/µl) at a cell density of 8000–10,000 cells per well. The final concentration of Endostar was 200 µg/ml. Cells were observed every 2 h by microscopy with $\times 100$ magnification for tube-like structure formation in 12 h. Three representative fields were taken and the average values of total tube length and tube formation area of each group were compared by Image-Pro Plus 6.0 (Media Cybernetics, USA).

Cell migration

Cell migration was examined with wound-healing assay in 12-well plates [8]. Cells were allowed to migrate for 8 h after

the center cells were removed. The final concentration of Endostar was 200 µg/ml. Cells were stained with crystal violet solution after being fixed with paraformaldehyde for further observation.

Cell invasion

Cell invasion was measured by transwell assays [9] using 8-µm pore transwells (Corning Costar, New York, USA) coated with ECM gel (1 µg/µl) in upper chamber of 24-well plates. Cells (2.5×10^4) were seeded onto the upper chamber of the transwells in RPMI-1640 containing 0.1% FBS. The final concentration of Endostar is 200 µg/ml in lower chamber.

Mice and murine xenograft lymphoma model

Four-week-old male BALB/c nude mice were purchased from the Jackson Laboratory (Vital River, Beijing, China). Animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Tongji Medical College. Mice were subcutaneously injected with 2×10^7 A20 cells into the right flank at Day 0. When the maximum diameter of tumor reached to 5 mm, mice were randomly grouped into blank group, placebo group, and Endostar group. The tumors were observed and measured every other day. The mice of Endostar group and placebo group received intraperitoneal injection of Endostar (2.5 mg/kg/day) or saline (0.2 ml/day), respectively, for 10 days.

Western blot analysis

Lysates were prepared from 1×10^7 cells by dissolving cell pellets in 100 µl of lysis buffer (Na₂HPO₄, pH 7.4, 20 mM, 150 mM NaCl, 1% Triton X-100, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 g/l leupeptin, 100 mM NaF, and 2 mM Na₃VO₄). Then lysates were centrifuged at 12,000 *g* for 15 min and the supernatant was collected. Protein content was determined using a Bio-Rad protein assay kit (Hercules, USA). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (10 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.2 M DTT) was added to the lysates. Lysates were heated at 100°C for 5 min, and 80 µg of protein was loaded into each well of a 10% SDS-PAGE gel. Resolved proteins were electrophoretically transferred onto nitrocellulose membranes, blocked with 5% non-fat milk or 5% BSA, and incubated with the primary antibodies (phospho-Akt: 1 : 500; phospho-ERK 1/2: 1 : 500; Akt: 1 : 1000; ERK1/2: 1 : 1000; and β-actin: 1 : 1000), respectively. After incubation at 4°C overnight, the membranes were washed, incubated with corresponding horseradish peroxidase-conjugated secondary antibodies for 1 h. After extensive wash, the blots were detected with ECL kit (Pierce, Rockford, USA) according to the manufacturer's protocol.

Statistic analysis

All data were expressed as the mean \pm SD of at least three separate experiments. $P < 0.05$ was considered to be statistically significant.

Results

Characteristic and identification of EPCs

EPCs were separated from human umbilical cord blood mononuclear cells. Colonies of endothelial cells appeared at Day 7, then expanded everyday and identified as monolayers of cobblestone appearing cells [Fig. 1(A)]. EPCs were identified by the uptake of acetylated low-density lipoprotein (red) and binding to lectin (green) like the other endothelial cells. HUVECs and EAhy.926 cells also showed the same characteristics [Fig. 1(B)]. EPCs have the ability to form tubes. In 2D and 3D culture model EPCs could form tube structure spontaneously [Fig. 1(C), left and Fig. 1(C), right].

Effects of endostar on the proliferation of EPCs, HUVECs, A20, and raji cells

EPCs, HUVECs, A20, and Raji cells were treated with different concentrations of Endostar (50, 100, 200, and 400 $\mu\text{g/ml}$) for 24, 48, and 72 h. The inhibition of EPC and HUVEC proliferation was in a dose- and time-dependent manner. There was no change in A20 and Raji cells. The OD value of Endostar-treated A20 and Raji cells groups decreased significantly compared with the EPCs and HUVECs groups. Results showed great difference among Endostar-treated EPCs, HUVECs, A20 cells, and Raji cells (Fig. 2). The IC_{50} of Endostar-treated EPCs for 24 h is 200 $\mu\text{g/ml}$. The proliferation of mature endothelial cells and EPCs could be inhibited by Endostar, but not for lymphoma cells.

Inhibition of angiogenesis in EPCs by endostar

EPCs were treated with 200 $\mu\text{g/ml}$ of Endostar on ECM gel for 12 h. The total tube length and tube formation area were less than the control group in phase-contrast microscopic images [Fig. 3(A)]. Scratch experiment was carried out to measure cell migration. Results showed that EPCs were migrated into scratch area in experimental group and were not migrated into scratch area in control group [Fig. 3(B)]. Transwell assay was used to measure cell invasion. EPCs were less at the bottom than that in control group when treated with Endostar (200 $\mu\text{g/ml}$) for 12 h [Fig. 3(C)].

Inhibitory effect of endostar on mice NHL tumor model

Murine B cell lymphoma transplant tumor model was successfully set up in this study. After being treated with Endostar for 10 days, the tumor volume was obviously diminished compared with the placebo group or the blank group. The tumor volumes in mice treated with Endostar for

20 and 24 days were decreased significantly compared with the control group (Fig. 4).

Expression levels of ERK, p-ERK, AKT, and p-AKT measured by western blot analysis

After being treated with 200 $\mu\text{g/ml}$ of Endostar for 24 h and stimulated by VEGF-A (15 ng/ml) for 30 min, the expression levels of AKT, ERK, and p-ERK in EPCs did not change, but the p-AKT expression was dramatically down-regulated. Its level was significantly decreased in Endostar group compared with the placebo group or the blank group. Results suggested that Endostar may inhibit the proliferation of EPCs by reducing the expression of p-AKT, but not p-ERK (Fig. 5).

Discussion

Tumor-associated neovasculature, generated by the process of angiogenesis, sustains expanding neoplastic growth in non-hematopoietic tumors. Tumor angiogenesis also exists in lymphoma development [10]. EPCs hold enormous potential to be used as a diagnostic and/or therapeutic agent in antitumor therapies due to their ability to self-renew, circulate, home to the ischemic sites, and differentiate into mature endothelial cells [11]. But the role of EPCs in lymphoma neovascularization remains poorly studied. EPCs can be divided into two types, the early EPCs and the late EPCs. Some researches called the late EPCs as EPC-derived endothelial cells (EPC-derived ECCs) or endothelial-colony-forming cells [12]. In our study, we chose EPCs of 1–6 generation as the experimental cells because of their higher capacity of proliferation and tube formation than those in the early EPCs.

Endostar, a traditional endostatin with an additional nine-amino acid sequence at the N-terminal of the protein and a His₆ tag, was reported to be more efficient in blocking angiogenesis and suppressing primary tumor and metastasis growth [13]. However, the effects of Endostar on NHL and EPCs are unclear. To explore whether Endostar inhibited proliferation of EPCs and NHL cells, we established the models of A20 NHL xenografts in nude mice and treated the mice with Endostar as described above. Taking into account that the majority of NHL mainly progresses as a systemic malignancy, our observation showed that the inhibitory effects of Endostar on NHL may inhibit the proliferation of mature endothelial cells and EPCs, but not the proliferation of Raji and A20 cells. Furthermore, our results indicated that the inhibitory effects of Endostar on tumor angiogenesis may also include the abilities to inhibit angiogenesis, migration, and invasion.

Many factors have been reported to play important roles in the mobilization of EPCs [14]. Among them, VEGF plays a very important role in cell mobilization and promoting angiogenesis [15]. Angiogenesis is very important in several

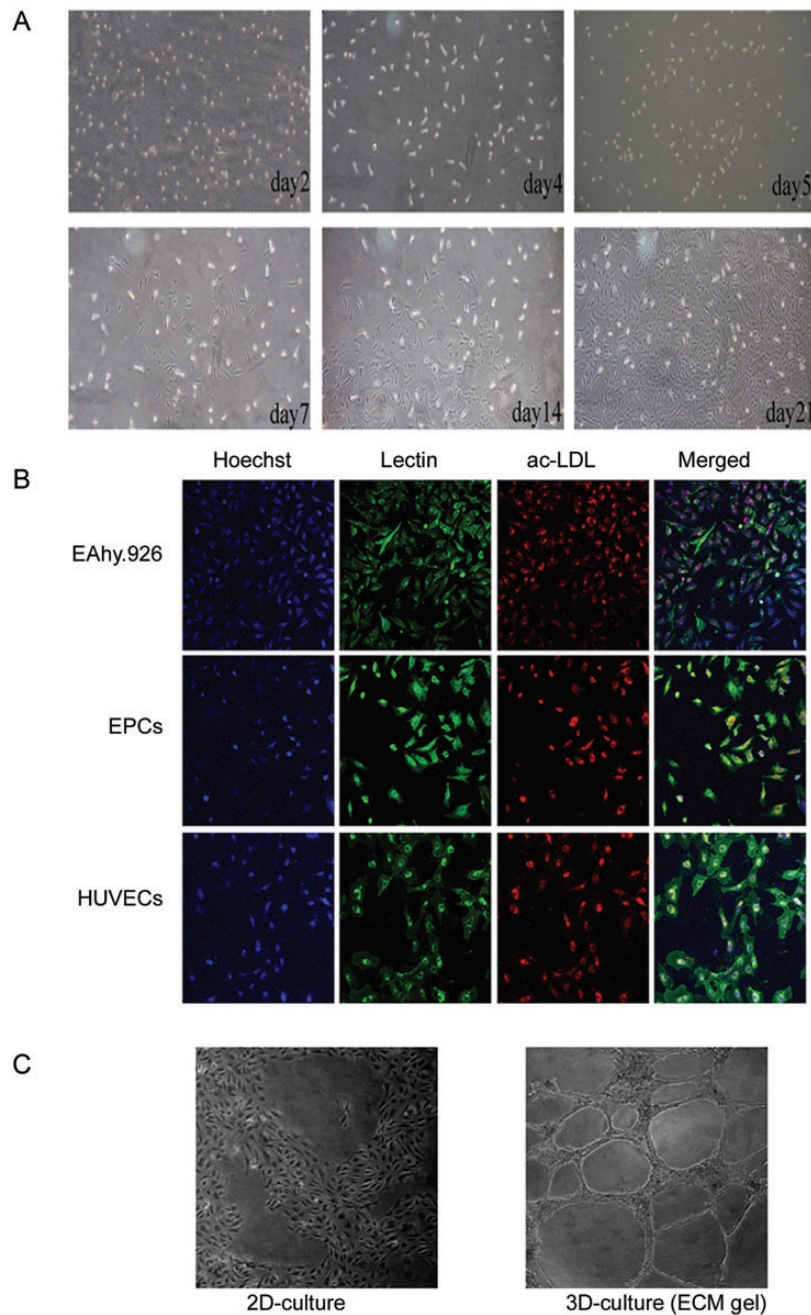


Figure 1 Characteristic and identification of EPCs (A) Phase-contrast images of sequential changes of EPCs in culture at different time intervals ($\times 100$ magnification). (B) Confocal microscopic images of immunofluorescent staining for the identification of HUVECs, EAhy.926, and EPCs. Green indicates FITC-labeled lectin; red indicates DIL-labeled ac-LDL; blue indicates nucleus ($\times 200$ magnification). (C) EPCs formed tube structure in 2D culture and 3D culture (left: $\times 100$ magnification; right: $\times 40$ magnification). $n = 3$.

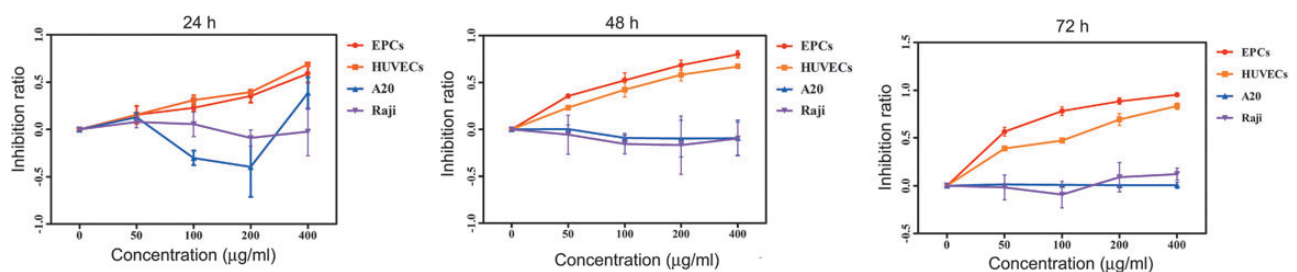


Figure 2 Effects of Endostar on the proliferation of EPCs, HUVECs, A20, and Raji cells by MTT assay Cells were treated with various concentrations of Endostar as indicated for 24, 48, and 72 h. Growth inhibition was determined using an MTT assay and shown as inhibitory rate. $n = 3$.

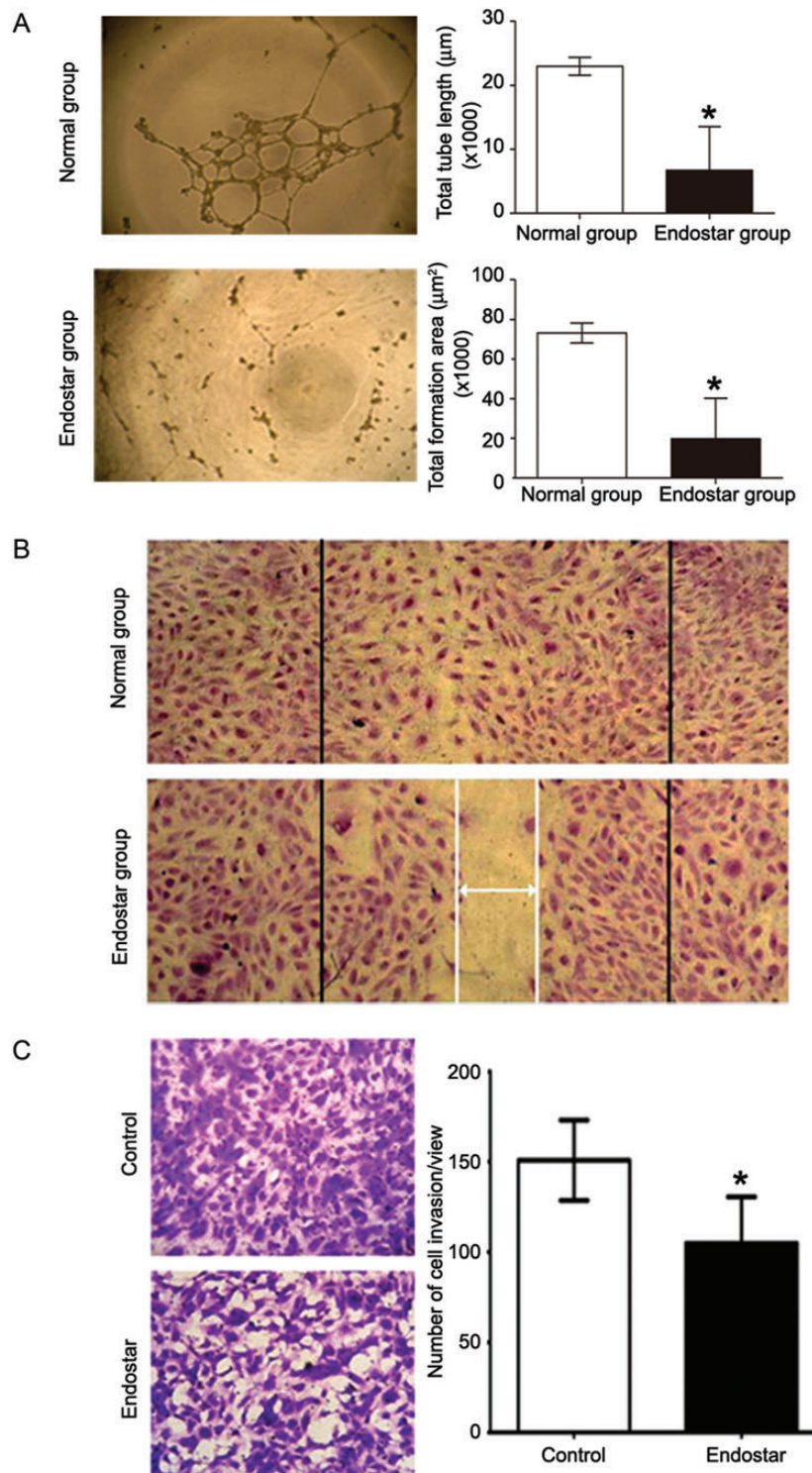


Figure 3 Endostar inhibits the EPCs capacity of angiogenesis (A) Phase-contrast microscopic images of the tube formation structures by EPCs and the control group on extracellular matrix (ECM) gel. EPCs were treated with 200 $\mu\text{g/ml}$ of Endostar for 12 h. The total tube length and tube formation area were less than the control group ($\times 100$ magnification). $n = 3$. (B) Cell migration assay with scratch experiment. EPCs were treated with 200 $\mu\text{g/ml}$ of Endostar for 12 h. EPCs were migrated into scratch area in experiment group and were not migrated into scratch area in the control group ($\times 400$ magnification). $n = 3$. (C) Cell invasion assay with by transwell. EPCs were treated with 200 $\mu\text{g/ml}$ of Endostar for 12 h. Representative phase-contrast microscopic images of transwell cell invasion were shown ($\times 100$ magnification). $n = 3$.

lymphoma subtypes. Increased serum and plasma VEGF concentrations have been linked to increased tumor burden, advanced stage and poor prognosis in patients with non-

Hodgkin's lymphoma. VEGF plays an important role in tumor angiogenesis, and due to lack of VEGF receptor (VEGFR) in tumor cell, VEGF produced by tumor cell

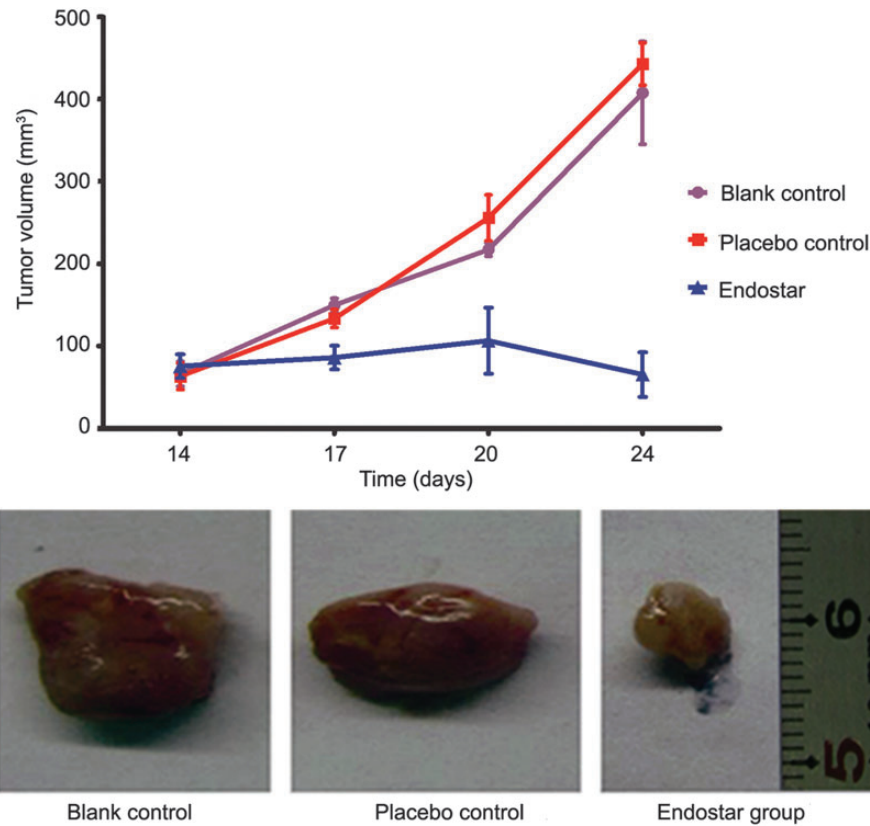


Figure 4 Inhibitory effect of Endostar on mice NHL tumor model The tumor volume of A20 mice tumor model was diminished after treatment with Endostar for 10 days. $n = 5$.

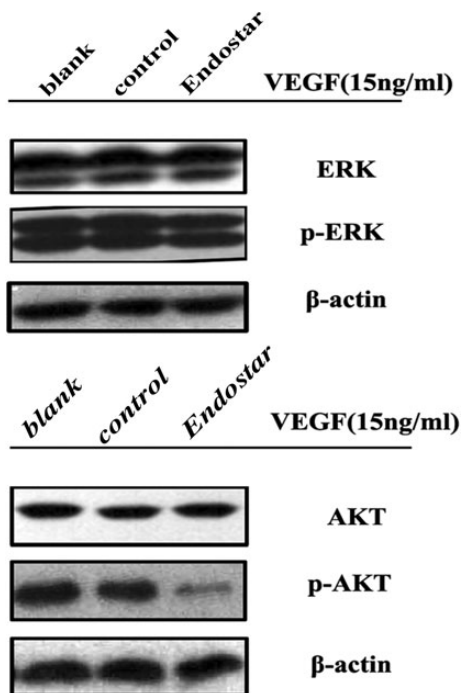


Figure 5 Effects of Endostar on the expression of ERK, p-ERK, protein kinase B (AKT), and p-AKT in EPCs Cells were treated with 200 $\mu\text{g}/\text{ml}$ of Endostar for 24 h at indicated time stimulating by VEGF-A (15 ng/ml). ERK, p-ERK, AKT, and p-AKT expression levels were measured by western blot analysis, respectively. $n = 3$.

paracrine mechanisms could not react with VEGFR [16]. On the other hand, EPCs produced a lot of VEGFRs that could react with VEGF and promote angiogenesis. The interaction of VEGF secreted from tumor cell with VEGFR secreted from endothelial cell cause a series of activities, including proliferation, tube formation, attack, and transportation capability [17–19]. It has been shown that the serum concentration of VEGF is correlated with the concentration of EPCs in tumor tissue [16,20]. However, how does VEGF mediate the mobilization of EPCs is still unknown. VEGF plays essential roles in vasculogenesis and angiogenesis [21]. Three main downstream pathways, ERK, PI3K, PKC pathways, are influenced by VEGF. The phosphatidylinositol 3-kinase (PI3K)/Akt and ERK pathways are the two important downstream pathways of VEGF [22]. In this study, we found that after being treated with Endostar and VEGF, the expression level of p-AKT was down-regulated, but p-ERK expression was not obviously changed. AKT, a serine/threonine protein kinase, was activated that led to the cell survival, proliferation, and cell metabolism [23]. PI3K pathway stimulates tumor-AKT angiogenesis and growth. p-AKT can prevent endothelial cell apoptosis, at the same time, activate the endothelial nitric oxide synthase and promote the endothelial cell migration and angiogenesis [24–26]. We found that Endostar may inhibit the proliferation of EPCs by reducing the expression of p-AKT, but not

reducing the expression of p-ERK. The decrease of p-AKT in EPCs highlights and provides a new field to explore the mechanism of Endostar.

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