·Basic Research·

Effect of nitric oxide derived from endothelial nitric oxide synthase (eNOS) on tumor angiogenesis

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[Abstract] Background and Objective: Nitric oxide (NO) derived from endothelial nitric oxide synthase (eNOS), which is expressed widely in tumor tissue, regulates tumor angiogenesis. However, the results are controversial. This study investigated the effect and the mechanism of NO derived from eNOS on tumor angiogenesis. **Methods:** C57BL/6 mice injected with Lewis lung cancer cells were randomized into three groups. In the NO group, mice were injected with lung cancer cells transfected with the eNOS gene. Tumor-bearing mice were treated with N(G)-nitro-L-arginine methyl ester (L-NAME), an eNOS antagonist, in the L-NAME group, or with normal saline in the control group. The plasma concentration of NO and the number of endothelial progenitor cells (EPCs) in peripheral blood were detected. To elucidate the involved mechanism, tumor vessel density, $CD133^+$ cells and the expression of VEGF–VEGFR in tumors were also measured. **Results:** At 4 weeks after the injection of the Lewis cells, tumor volume in the control group was (3022 ± 401) mm^3, while the tumor volumes were (1204 ± 97) mm^3 and (1824 ± 239) mm^3 in the L-NAME group and the eNOS group, respectively ($P < 0.01$). Proteins of eNOS and NO production increased significantly in Lewis lung cancer cells transfected with eNOS gene. But the number of CD133-positive cells and the vessel density in tumors were significantly lower in the eNOS group [(48 ± 19) per high-powered field (/HPF) and (19 ± 7)/HPF, respectively] than in the control group ($P < 0.05$). There was no statistical difference in the number of EPCs in peripheral blood between each group. The concentration of NO in blood and tumor tissue was significantly decreased by L-NAME, while the tumor vessel density reduced to (12 ± 5)/HPF ($P < 0.01$ vs. the control group; $P < 0.05$ vs. the eNOS-transfected group). The number of EPCs in the blood and CD133-positive cells in tumor tissue also decreased in the L-NAME group compared with the control group ($P < 0.05$). **Conclusions:** Our results demonstrate that NO derived from eNOS inhibits tumor growth via regulating angiogenesis, which may be due to its suppression on either the mobilization or homing of EPCs via VEGF binding to VEGFR.

Key words: Endothelial nitric oxide synthase, nitric oxide, endothelial progenitor cells, tumor angiogenesis, vascular endothelial growth factor

Nitric oxide (NO) is a multifunctional low-molecular weight gas molecule synthesized with L-arginine and oxygen as catalyzed by NO synthase (NOS). With considerable activity as a free radical, it is involved in numerous physiologic and pathophysiologic processes, such as angiogenesis. There are three subtypes of NOS, namely neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). In recent years, studies have found that not only was eNOS expressed in normal tissue, it was also extensively expressed in tumor tissue.[1] By catalyzing the synthesis of NO and regulating the breakdown of endothelial cells[2] and the mobilization and homing of endothelial progenitor cells (EPCs) in bone marrow,[3] it participates in angiogenesis and therefore influences the growth and metastasis of tumors. Therefore, intervention against NOS activity and NO synthesis may become a treatment strategy for tumors.

However, a large number of studies have yielded inconsistent results, complicating the focus for researchers. For example, the positive expression rate of eNOS was 89% in non-small cell lung cancer tissue, and patients with a strong-positive expression had better survival rates than those with weak expression,[4] indicating that the NO catalyzed by eNOS inhibited tumor growth. However, knockdown of the eNOS gene or using a selective inhibitor of eNOS [N(G)-nitro-L-arginine methyl ester (L-NAME)] also inhibited

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This paper was translated from Chinese into English by Guangzhou Liheng Medical Translation and edited by Hope J. Lafferty on 2009-10-09.

The Chinese version of this paper is avaiable at: http://www.cjcsysu.cn/cn/article.asp?id=16179.

Grants: Foundation of Health Department of Sichuan Province (No. 070028)

Received: 2009-05-06; Accepted: 2009-09-23

the homing of peripheral EPCs[5] and neoangiogenesis in tumors,[6] demonstrating that NO promoted neoangiogenesis.

NO is an active substance of small molecular weight with a significantly dose-dependent effect: at lower concentrations, NO reduces the tissue damage from O2- by reacting with it;[7] but at higher concentrations, NO can result in direct damage on the tissue.[8] Is neoangiogenesis also dependent on the concentration of NO? Using mouse models of Lewis lung cancer, our study set to explore its regulation mechanism and whether the regulation on neoangiogenesis by the NO catalyzed by eNOS was concentration-dependent.

Materials and Methods

Plasmid construction, transfection, and verification of tumor cells

The cDNA of the eNOS gene (by Invitrogen) was amplified by polymerase chain reaction (PCR). Primers for the PCR amplification were 5'-ATCTGATGCTGCC-3' (upstream) and 5'-GTTACTGTGCGT-3' (downstream) (Shanghai GeneCore Biotechnologies Co., Ltd). Amplified eNOS DNA segments were ligased to the EcoRI site of the eukaryotic expression vector pcDNA3.1 (Number: V795-20; Invitrogen) to construct a recombinant plasmid pcDNA3.1-eNOS (9 kb), which was then verified by endonuclease *Eco*R I and *Xho* I. Cells were then transfected with the plasmid pcDNA3.1-eNOS and the empty vector pcDNA3.1 purified using a purification reagent kit (Qiagen). DNA concentration in the plasmid was determined by ultraviolet spectrophotometer. Purified plasmid was preserved at -20℃ for subsequent experiments.

Lewis lung cancer cells (the cell line was offered by the State Key Laboratory in West China Hospital of Sichuan University) were injected into 6-well plates. When the cells were 60% –70% confluent, they were transfected according to the protocol in the Lipofectin transfection reagent kit. Cells were collected from each well and combined with 2 μg of either pcDNA3.1-eNOS plasmid or empty plasmid and 4 μg of Lipofectin (Invitrogen, San Diego, CA, USA), and were then injected into serum-free Dulbecco's modified eagle medium (DMEM) (Gibco-BRL). After incubating at 37℃ for 12 h, the medium was replaced by serum containing DMEM. The incubation proceeded for another 60 h, and then the supernatant fluid was collected and preserved at -80℃ for the detection of the eNOS protein. When the transfected cells were in the stationary phase, two types of transfected cells were collected at the logarithmic growth phase and injected into the mice.

At the same time, untransfected Lewis lung cancer cells were incubated and an equal number of cells was collected at the logarithmic growth phase. Mouse enzyme-linked immunosorbant assay for eNOS (eNOS-ELISA) reagent kits were purchased from USCN Life Science Inc. At 72 h after the transfection of the Lewis cells, the cells were collected and centrifuged. The precipitation was collected, redissolved, and centrifuged again. The supernatant fluid was obtained for the detection of eNOS protein expression as described in the protocol of the reagent kit. Lewis cells were incubated at 37℃ and 5% CO_2 and collected at the logarithmic growth phase.

Establishing the tumor model, group assignment, and treatment

After centrifugation, the cell precipitation was washed and then injected subcutaneously into the right flank of female C57BL/6 mice of 6-8 weeks of age and around 20 g in weight (certificate number: 0006549) to establish the tumor models (1×10^6 cells for each mouse). The mice were randomly divided into three groups, with ten mice in each group. In the transfection group, the mice were injected with Lewis cells in the stationary phase after being transfected with pcDNA3.1-eNOS. In the L-NAME group, the mice were injected with Lewis cells that were incubated as usual for 7 days (tumor diameters were about 0.5 cm) and then received intraperitoneal (IP) injections of L-NAME [20 mg/(kg·d)]. In the control group, the mice were injected with Lewis cells that were cultured as usual for 7 days and then received IP injections of an equal amount of normal saline. At the end of these treatments, the mice were observed for 3 weeks, during which the tumor volume (length × width^2 × 0.52) was measured every 4 days. At the end of the observation, peripheral blood and tumor tissue were obtained from the mice for detection.

The detection of plasma NO product and concentrations of vascular endothelial growth factor (VEGF)

Blood samples of the mice were drawn by a terminal cardiac puncture after opening of the chest wall and collected in heparin anticoagulated tubes. After centrifugation at 311 × *g* for 10 min, the supernatant fluid (plasma) was retained for subsequent experiments. Since the half-life of NO is no more than a few seconds, we detected the concentration of an NO metabolic product, nitrite, as a surrogate for NO production. Plasma NO detection reagent kits (nitrate reductase method) were purchased from Nanjing Jiancheng Bioengineering Institute, and detection was conducted as described in the protocol of the reagent kit.

Flow cytometry for the detection of blood EPC

Detection of EPC was performed using direct labeling. Nucleated cells from the blood were prepared into single-cell suspension. A group of 1×10^6 cells were washed by phosphate-buffered saline (PBS) and combined with fluorescein isothiocyanate [FITC (CD133)], phycoerythrin [PE (CD34)], and PE-CY7 (KDR) antibodies (BD Biosciences) to label antigens on the cell membrane. At the same time, a negative tube was prepared as the control. The cells were then incubated at room temperature for 20 min and washed with PBS again. The supernatant fluid was removed, and the cells were resuspended with 300 μL PBS and used for detection.

Measurement of eNOS protein expression in tumor tissue

The expression of the eNOS protein in tumor tissue was detected using ELISA. An amount of 0.4 mg of tumor tissue was obtained and homogenated by ultrasound in an ice bath. The homogenate was centrifuged at 10 000 × *g* at 4℃ for 20 min. The supernatant fluid was obtained and used for eNOS detection in accordance with the protocol in the reagent kit.

The detection of vessel density in tumor tissue

The detection was conducted in accordance with the methods described in the literature:[9] with avidin biotin complex (ABC) immunohistochemical staining (Vector Laboratories), the CD31 antibody was used to stain microvessels in frozen sections of tumor tissue. A total of ten visual fields were randomly selected from each section and observed by specialists in a double-blind fashion. The number of microvessels were counted for each section and the mean number of microvessels/HPF were calculated as a marker for microvessel density in tumor tissue. Purified rat anti-mouse CD31 (PECAM-1) monoclonal antibody was purchased from BD Bioscience.

CD133$^+$ cell staining and the VEGF-VEGFR complex in tumor tissue

CD133$^+$ cells were stained using immunofluorescence. The primary antibody (goat anti-mouse CD133 antibody) was purchased from Santa Cruz and the secondary antibody (swine anti-goat) from SBA. To investigate the mechanism underlying the influence of NO on the homing of CD133$^+$ cells, we used a specific antibody of the VEGF-VEGFR2 (vascular endothelial growth factor receptor 2) complex, GV39M,[10] and ABC immunohistochemical staining to label the VEGF-VEGFR2 complex. The measurement went according to the description in Subsection 1.5. The respective antibodies were purchased from BD Bioscience and EastCoast Bio.

Statistical analyses

SPSS13.0 software was used for the statistical analyses. All data were shown as mean value standard deviation (mean SD). Comparisons between groups were analyzed with a *t* test. $P < 0.05$ suggested statistical significance.

Results

The expression of eNOS in transfected cells

When Lewis lung cancer cells were transfected, the expression of the eNOS protein significantly increased as compared to the control group.

Changes in tumor volume

As shown in Figure 1, tumor volume increased to (3022 ± 401) mm^3 in the control group at 4 weeks after tumor-cell injection, while in the L-NAME and the eNOS groups, the tumor volumes were (1204 ± 97) mm3 and (1824 ± 239) mm^3, respectively, with significant differences compared to control group ($P < 0.01$). These findings indicated that both the inhibition of NO production by L-NAME and the increase in NO production by eNOS transfection could inhibit tumor growth.

eNOS expression in tumor tissue and plasma concentration of NO

In the control group, eNOS protein concentration in tumor tissue was (105 ± 36) pg/mg and the NO concentration in plasma was (10.5 ± 4.2) μmol/L. Figure 2 shows that the IP injection of L-NAME failed to change the eNOS protein concentration in tumor tissue, but could significantly inhibit NO production [(3.8 ± 1.9) μmol/L; $P < 0.01$]. When tumor cells were transfected with the eNOS gene, the eNOS protein concentration in the tumor tissue increased to (382 ± 75) pg/mg, and the plasma NO concentration also increased to (19.7 ± 3.6) μmol/L, which were significantly higher than the control group ($P < 0.01$).

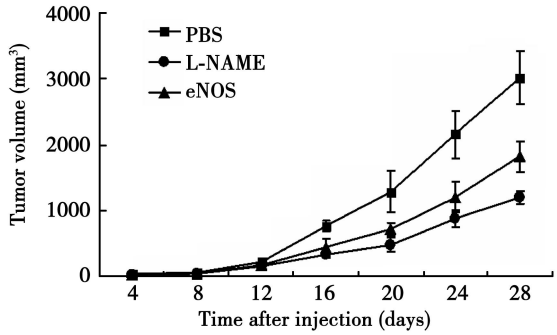

Figure 1 Growth inhibition of Lewis lung cancer

Mice carrying subcutaneous Lewis lung cancer cells were treated with PBS (squares, *n*= 10) or L-NAME (circles, *n*=10). Mice carrying Lewis lung cancer cells tranfected with eNOS gene (triangles, n=10) did not receive any treatment. Both treatment with L-NAME and the transfected eNOS gene resulted in significant inhibition of tumor growth vs controls.

Microvessel density in tumor tissue

Figure 3 shows that the mean number of microvessels in the control group was (31 ± 9)/HPF, which was significantly higher than the eNOS transfection group (19 ± 7)/HPF ($P < 0.05$). The L-NAME group had the lowest microvessel density, (12 ± 5)/HPF, with statistically significant differences when compared to both the

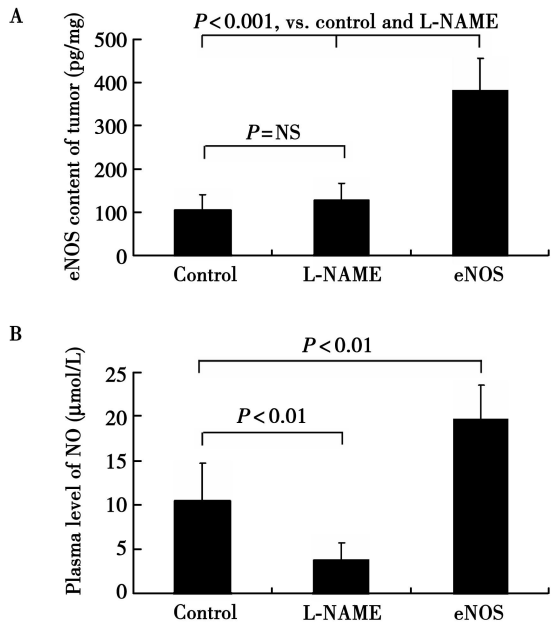

Figure 2 eNOS content in tumor tissue and nitric oxide concentration in plasma of three groups of mice

C57BL/6 mice were randomized into three groups: the eNOS group (injected with Lewis lung cancer cells transfected with the eNOS gene); the L-NAME group (tumor-bearing mice received IP injections of the eNOS agonist); and the control group (injected with the same volume of normal saline). Compared with the control group, increased eNOS content in tumor tissue ($P < 0.001$, A) and nitric oxide in plasma ($P < 0.01$, B) were only seen in the eNOS group after treatment for 3 weeks. In the L-NAME group, plasma concentrations of NO decreased ($P < 0.01$).

control and the eNOS transfection groups ($P < 0.05$).

Endothelial progenitor cell counts in circulation

In this study, EPCs were labeled using $CD133^+$, $CD34^+$, and KDR+. As shown in Figure 4, EPC count in peripheral circulation was (34 ± 5)/mL of whole blood in the control group. When treated with L-NAME, the EPC count was (18 ± 4)/mL, with a statistically significant difference compared to the control group ($P < 0.05$). The circulating EPC count was (26 ± 6)/mL in the eNOS transfection group, with a statistically insignificant difference when compared to the control group ($P > 0.05$).

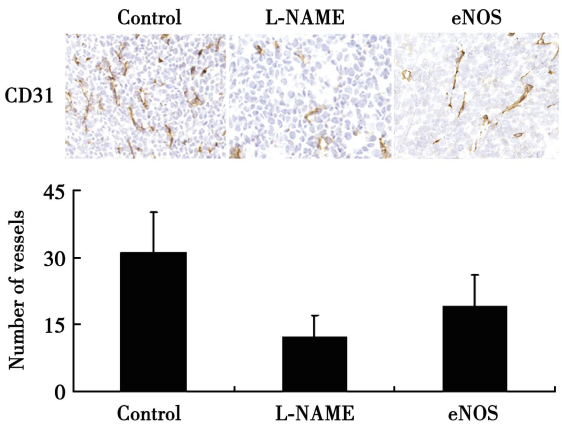

Figure 3 Microvessel densities of tumor tissue (IHC ABC × 400)

C57BL/6 mice were randomized into three groups: the eNOS group (injected with Lewis lung cancer cells transfected with the eNOS gene); the L-NAME group (tumor-bearing mice IP injected with the eNOS agonist); and the control group (injected with the same volume of normal saline). Microvessel densities of tumor tissue of both the L-NAME and the eNOS groups were significantly lower compared with the control group ($P < 0.01$ and $P < 0.05$, respectively).

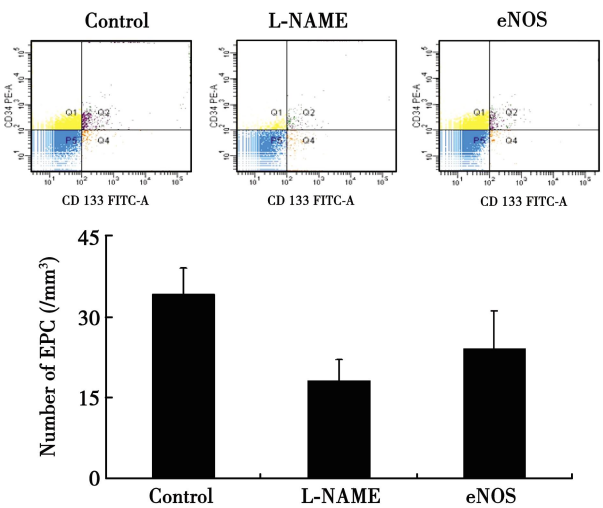

Figure 4 The number of EPCs in peripheral blood circulation

The number of EPCs in peripheral blood circulation in the L-NAME group decreased compared with the control group, ($P < 0.01$). There was no difference of the number of EPCs between the eNOS group and the control group ($P > 0.05$).

Homing of CD133+ cells in tumor tissue

CD34 and KDR expressions can be seen even on mature endothelial cells, so in this study we used the CD133 antigen to label homing EPCs in tumor tissue. As shown in Figure 5, the homing EPC count in the tumor tissue of the tumor-bearing mice without any treatment was (103 ± 27)/HPF. When tumor-bearing mice received IP injections of L-NAME, the EPC count in tumor tissue was (29 ± 11)/HPF ($P < 0.01$ vs. the control group). When the mice were injected with the Lewis lung cancer cells that had been transfected with eNOS, the EPC count in the tumor tissue was (48 ± 19)/HPF, which was significantly lower than the control group ($P < 0.01$), but higher than the L-NAME group ($P < 0.05$).

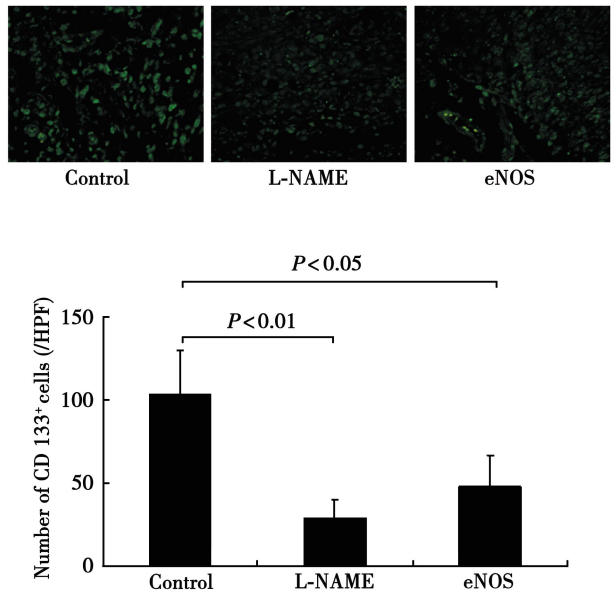

Figure 5 The number of $CD133^+$ cells in tumor tissue by immunofluorescence (×400)

The number of $CD133^+$ cells in tumor tissue decreased by treatment with both L-NAME and eNOS transfection compared with normal saline treatment in the control. ($P < 0.01$ and $P < 0.05$, respectively).

VEGF expression in the circulation and expression of the VEGF-VEGFR2 complex in tumor tissue

Immunohistochemical staining showed that the VEGF-VEGFR2 complex was expressed at different levels in tumor tissue of various groups of animals, while in the L-NAME and the eNOS transfection groups, expression of the VEGF-VEGFR2 complex was lower than the control group (Figure 6A).

ELISA detection showed that the concentration of VEGF in plasma was similar in the animals of the control and the eNOS transfection groups [(47.5 ± 12) pg/mL vs. (48.3 ± 18) pg/mL, $P > 0.05$]; whereas the concentration in the L-NAME group was (22.5 ± 7) pg/mL, which was significantly different as compared to the two other groups ($P < 0.05$) (Figure 6B).

Discussion

Tumor growth and metastasis are dependent on tumor angiogenesis. Anti-angiogenic treatment has become an important therapeutic strategy to treat tumors. NO is involved in the neoangiogenesis process in tumors,[1] and may be a target for anti-angiogenesis treatment. Therefore, the mechanism that

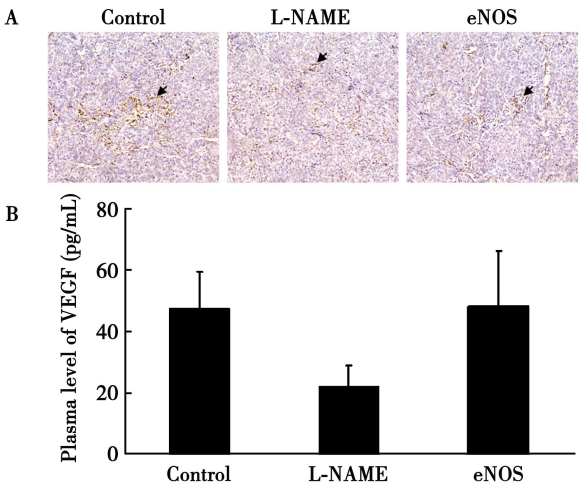

Figure 6 VEGF concentration in plasma and the VEGF-VEGFR$_2$ complex in tumor tissue (IHC ABC ×400)

The complex of VEGF–VEGFR2 (A, arrow) in tumor tissue was significantly higher in the control group than in the L-NAME and eNOS groups. However, plasma VEGF levels (B) were similar in the control and eNOS groups, and were significantly higher in the control and eNOS groups compared with the L–NAME group ($P < 0.01$).

influences NO on tumor angiogenesis has become an important research topic. However, the effects of NO on tumors are subject to the influences of NO catalysts, concentrations, and types of tumors,[1] and the mechanism is still unclear. Our study found that the use of the eNOS-inhibitor L-NAME reduced NO concentration in peripheral circulation, and the microvessel density in tumor tissue decreased accordingly. When tumor cells transfected with the eNOS gene were injected, the lung cancer tissue that was developed subsequently had lower microvessel density than the non-transfection group, indicating that both extremely high and extremely low concentrations of NO could impact tumor angiogenesis.

Unlike normal vessels, tumor vessels have disconnected endothelia. Still, the extension of the vascular endothelium is an important prerequisite for angiogenesis.[11] Extending endothelial cells may originate from the division of adjacent endothelial cells and circulating EPCs. Since the adjacent cells are mature endothelial cells without much mitotic activity, EPCs may play a critical role in angiogenesis.[2,4] Deriving mainly from bone marrow, EPCs are progenitor cells of mature endothelial cells that regulate vascular repair and neoangiogenesis.[11,12] In 2006, Shaked *et al.*[13] found that large amounts of EPCs were released from the bone marrow when tumors were destroyed by chemotherapeutic agents. These EPCs rapidly gathered around the margin of the tumor and constructed new vessels, indicating that mobilization and homing of EPCs was an important condition for neoangiogenesis.

Is NO involved in the mobilization and homing of EPCs and thereby regulating angiogenesis in the tumor? EPC expresses $CD133^+$, $CD34^+$, and VEGFR2+ (KDR) on the cell surface. In this study, we used flow cytometry to detect EPCs in peripheral circulation, and immunohistochemical staining to label $CD133^+$ EPCs in tumor tissue. The results revealed that, when tumor-bearing mice received IP injections of the eNOS inhibitor L-NAME, the EPC count in blood circulation reduced from 34 cells/mL of whole blood to 18 cells/mL of whole blood as the serum NO concentration and the NO concentration in tumor tissue decreased. $CD34^+$ cell count in tumor tissue significantly decreased as well. These findings suggest that the decrease in NO production as catalyzed by eNOS could reduce both mobilization and homing of EPCs and thus lessen tumor angiogenesis.

Although the current evidence is not enough to support that NO catalyzed by eNOS can directly induce the mobilization and homing of bone marrow-derived EPCs, there is evidence suggesting that NO has an important role in the EPC mobilization and homing induced by VEGF.[2,3] VEGF binds to the receptor VEGFR2 (KDR) on target cells and increases the chemotaxis in EPCs, promotes cell division and budding and growth of capillary vessels, and enables endothelial cells to penetrate the stroma and create new vessels.[12,14] When the eNOS gene is knocked down, VEGF injection can no longer increase the homing EPC count in either peripheral circulation or ischemic tissue,[15] suggesting that NO is an important substance in regulating the angiogenesis induced by VEGF. Does NO regulate the mobilization and homing of EPCs via VEGF? Our study revealed that IP injection of the eNOS-inhibitor L-NAME in tumor-bearing mice led to decreased NO production in both the bloodstream and the tissue. Plasma VEGF concentration was reduced, while the expression of VEGF-VEGFR2 in tumor tissue significantly decreased accordingly. These demonstrated that decreased NO concentrations resulted in less EPC mobilization and homing by either reducing VEGF synthesis and release or inhibiting the binding of VEGF to its receptors.

The NO catalyzed by eNOS can promote tumor angiogenesis and increase the blood supply for tumors, and thus favor the growth and metastasis of tumor cells. However, our study showed that, when Lewis lung cancer cells transfected with the eNOS gene were subcutaneously injected into mice for tumor development, the NO metabolic product increased. No influence was seen for the concentration of VEGF in plasma, but the $CD133^+$ cell count and expression of VEGF-VEGFR2 in tumor tissue were significantly decreased, indicating that abnormally increased NO could also inhibit EPC homing by preventing VEGF from binding to its receptor. The mechanisms might be: (1) high concentrations of NO and its metabolic product act as free radicals and thus impair the activity of VEGF; (2) by changing the intracellular signal transduction pathway for circulating EPCs, it inhibits the locomotion, homing, and proliferation of EPCs;[16,17] or (3) it is cytotoxic and promotes apoptosis in EPCs. However, the exact mechanisms should be further investigated.

Our study also found that when eNOS was transfected into tumor cells, the circulating EPC count was not changed even though the serum NO metabolic product increased. This might be because that the half-life of NO was so short (no more than a few seconds) that it was impossible for the NO generated in tumor tissue to reach the bone marrow, and thus it had no influence on EPC mobilization.

In conclusion, by regulating the activity of eNOS, the mobilization and homing of EPCs induced by VEGF could be inhibited by both decreased and increased NO production, and neoangiogenesis in Lewis lung cancer was thus inhibited. This demonstrated that NO concentration might be an important regulator for tumor angiogenesis. Therefore, therapy targeting eNOS or NO might become another strategy in antitumor treatment.

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