Angiogenesis Inhibitor, Endostar, Prevents Vasa Vasorum Neovascularization in a Swine Atherosclerosis Model

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Aim: Vasa vasorum neovascularization is a key feature of atherosclerosis (AS) and is strongly associated with inflammatory infiltration, lipid deposition, intraplaque hemorrhage, and hemosiderin deposit. Here we investigate the effects of Endostar, a strong anti-angiogenic drug, on vasa vasorum neovascularization in the experimental porcine model of early AS.

Methods: Eighteen adult male Ba-Ma mini pigs were randomized into three groups, with six animals in each group. The pigs in the normal (N) group were fed a normal diet for 18 weeks, without balloon injury surgery. The animals in the atherosclerotic (AS) control and AS+Endostar groups were fed a hypercholesterolemic diet for 12 weeks after balloon injury surgery; they received either saline or Endostar for an additional six weeks, while continuing the hypercholesterolemic diet. The atherosclerotic abdominal aorta and levels of serum lipids, TNF-alpha, IL-6, and hs-CRP were analyzed at 18 weeks.

Results: The AS group had a significantly higher body weight and serum lipid concentration levels than the N group (p<0.05), confirming the success of the hypercholesterolemic diet. However, no statistical differences were noted between the AS and AS+Endostar groups. Histopathology results revealed that vasa vasorum density and intima-media thickness (IMT) had also increased in the AS group compared with those in the N group (p<0.05). The Endostar treatment significantly alleviated AS with decreased vasa vasorum density and IMT (AS vs. AS+Endostar, p<0.05). Western blot analysis indicated that the expression of VEGF, β-catenin, and TNF-alpha in the atherosclerotic abdominal aorta was considerably reduced by the Endostar treatment. In addition, immunohistochemistry results showed that the angiogenesis markers VEGF and β-catenin were predominately localized in endothelial cells of the adventitial vasa vasorum. The levels of the serum inflammatory markers TNF-alpha, hs-CRP, and IL-6 were markedly higher in the AS group than in the N group (p<0.05) but showed no marked difference during the Endostar treatment, suggesting that the local inhibition of angiogenesis was not accompanied by a change in serum inflammatory markers and that the inhibitive effect of Endostar on local TNF-alpha expression could be because of the prevention of vasa vasorum neovascularization.

Conclusions: Our results demonstrated that the Endostar treatment inhibited vasa vasorum neovascularization and AS progression in the experimental porcine model of early AS, supporting the role of vasa vasorum neovascularization in the development of AS and the therapeutic potential of anti-angiogenesis intervention in AS.


Key words: Atherosclerosis, Vasa vasorum, Neovascularization, Endostar, Swine
**Introduction**

Coronary artery disease (CAD) because of atherosclerosis (AS) is a leading cause of morbidity and mortality worldwide. AS is a commonly recognized inflammatory disease, although the mechanism of its initiation and progression is not fully understood.

Previous studies have shown that vasa vasorum neovascularization increases in both early and advanced AS. Vasa vasorum neovascularization is also strongly associated with inflammatory infiltration, lipid deposition, intraplaque hemorrhage, and hemosiderin deposit. Recent clinical research have shown that vasa vasorum rupture and intraplaque hemorrhage may lead to plaque destabilization by accumulating macrophages and enlarging the lipid/necrotic core. These observations suggested that vasa vasorum neovascularization plays an important role in the progression and complications of AS. Furthermore, current studies have demonstrated that the inhibition of vasa vasorum neovascularization attenuates the development of AS, suggesting that it is a potential strategy for preventing the disease.

Endostar, a novel recombinant human endostatin with an N-terminal modification, has a broad spectrum of anti-angiogenic functions and was approved by the State Food and Drug Administration of China (SFDA) for the treatment of non-small-cell lung cancer in 2005. Endostar is also a specific angiogenesis inhibitor that suppresses pathological angiogenesis and has no activity against wound healing. Compared with previously reported endostatin, the half-life of Endostar is much longer. As a functional angiogenesis inhibitor, Endostar is at least twice as efficient as endostatin in animal tumor models. It also reduces inoculated tumor growth in mice by substantially inhibiting angiogenesis. In the recent pilot clinical trial, Endostar, combined with chemotherapy, was well tolerated in patients with metastatic colorectal and gastric cancers. Finally, our previous study has revealed that Endostar can inhibit neovascularization and plaque growth in the rabbit AS model.

Although a lot of insight into molecular and disease processes has been gained from small animal models, including murines, tremendous anatomic and physiological differences still exist between these models and humans. Therefore, large animal models are essential for developing the discoveries from smaller ones into clinical therapies and interventions for humans. Balloon injury combined with dietary hypercholesterolemia is a standard method for inducing AS in animals, which mimics the pathological mechanism of AS in human beings. In this study, we used balloon injury and a hypercholesterolemic diet to develop an AS model in swine. Furthermore, using a new angiogenesis inhibitor, Endostar, we tested the prevention of vasa vasorum neovascularization in this AS porcine model.

**Methods and Materials**

**Animal Models and Specimen Acquisition**

All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals, and all protocols were approved by the Animal Ethics Committee of Zhejiang Chinese Medical University, Hangzhou, China. Healthy male Ba-Ma mini pigs (6 months old; weight, 10–15 kg) were purchased from the Zhejiang Chinese Medical University Animal Experiment Center. The porcine AS model was induced by balloon injury combined with a hypercholesterolemic diet, as previously described with modifications. Eighteen healthy male Ba-Ma mini pigs were randomized into a normal group (N group, n = 6), an atherosclerotic model group (AS group, n = 6), and an Endostar (Shan-dong Xiansheng Maidejin Biological Pharmaceutical Co., Ltd, Shandong, China)-treated group (AS + Endostar group, n = 6). All groups were initially fed a normal diet. The AS and AS + Endostar groups were deprived of food for 12 h and were then given an injection of ketamine (3 mg/kg). An anesthesia ventilator (AS-01-0001, Beijing GYD Labtech Co., Ltd, Beijing, China) was used for assisted breathing (tidal volume, 12–15 mL/kg, respiratory rate: 15–18 times/min). Each pig was maintained on isoflurane inhalation anesthesia (1.5%–2%), and physiological and hematological parameters were measured. Further, the femoral artery was separated under aseptic technique, and a special artery sacculus tube (8 × 40 mm, Medtronic, Minneapolis, MN, USA) was inserted to 35 cm in the abdominal aorta. The balloon was injected with saline to create 12-atm (1 atm = 1.01325 × 10^5 Pa) pressure and was then slowly pulled back and forth for five times. Finally, the femoral artery was ligated, and the incision was sewn up by layers. After the operation, each pig was injected with benzylpenicillin sodium (8,000 U/day) for three continuous days. The AS and AS + Endostar groups were fed a hypercholesterolemic diet (1.5% cholesterol, 0.5% sodium cholate, 0 extracted cholesterol, and 0.25% lard) for 4 weeks. Each pig was injected with benzylpenicillin sodium (8,000 U/day) for three continuous days. The AS and AS + Endostar groups were fed a hypercholesterolemic diet (1.5% cholesterol, 0.5% sodium cholate, and 0.25% lard) for 4 weeks.
15% oleomargarine, 10% egg yolk powder; CML005, 22 g/kg, Zhejiang Chinese Medical University Animal Experiment Center, Hangzhou, China) for 12 weeks, while the N group was still fed a normal diet; the histopathology was then assessed. Further, the AS and AS+Endostar groups were injected with saline and Endostar 7.5 mg/ (m²·d), respectively, according to the manufacturer’s recommendations. After six weeks of treatment, the animals were euthanized. The atherosclerotic abdominal aorta (approximately 4-cm long, below the renal artery) was then carefully collected and divided into two parts at approximately 2 cm below the renal artery (the maximal stenosis area). One part was freshly frozen in liquid nitrogen for western blot, while the other part was fixed in formalin and embedded in paraffin for histology and immunohistochemistry.

**Histology and Immunohistochemistry**

Cross sections of the abdominal aorta (5-μm thick, 2 cm below the renal artery) were obtained with a microtome (Leica RM2135) and mounted on slides. After deparaffinization and rehydration, all sections were stained with hematoxylin–eosin and Verhoeff’s Van Gieson elastin stains. The thicknesses of the intima and media were measured using the Image-Pro Plus 5.1 image operation system. For immunohistochemistry, the sections (5-μm thick, 2 cm below the renal artery) were first deparaffinized and rehydrated. After the endogenous peroxidase activity was blocked with 3% H₂O₂, the antigen was retrieved by quickly heating. Primary antibodies [Anti-von Willebrand factor (vWF) antibody, ab68545, 1:100, Abcam, Hong Kong; Anti-β-catenin antibody, ab23671, 1:100, Abcam, Hong Kong; anti-vascular endothelial growth factor (VEGF) antibody, NB100-648, 1:100, Novus Biologicals, Cambridge, United Kingdom; Anti-TNF-alpha antibody, ab6671, 1:100, Abcam, Hong Kong; Anti-β-actin antibody, A2228, 1:1000, Sigma, St. Louis, MO] and were then exposed to the corresponding secondary antibody [Anti-rabbit or -mouse IgG (H+L) antibody, 7074S, 7076S, Cell Signaling Technology, Danvers, MA]. Protein bands were detected with an enhanced chemiluminescent substrate (EZ-ECL, Biological Industries, Kibbutz Beit-Haemek, Israel), scanned, and quantified by a Bio-imaging analyzer (Bio-Rad).

**Western Blot**

To determine protein expression levels of β-catenin, VEGF, and tumor necrosis factor (TNF)-alpha, abdominal aorta tissues (2 cm below the renal artery) were homogenized by a tissue homogenizer and ultrasonic processor in RIPA lysis buffer (P0013C, Beyotime, China) with freshly added phenylmethanesulfonyl fluoride (ST506, Beyotime, China). The lysate protein content was quantified by a Bio-Rad Protein Assay Kit (500-0006; Bio-Rad). A total of 30 μg protein samples were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (IPVH00010; Millipore) by Trans-Blot® SD Semi-dry Electrophoretic Transfer (170-3940; Bio-Rad). Transferred membranes were incubated with primary antibodies (Anti-β-catenin antibody, ab23671, 1:100, Abcam, Hong Kong; Anti-VEGF antibody, NB100-648, 1:100, Novus Biologicals, Cambridge, United Kingdom; Anti-TNF-alpha antibody, ab6671, 1:100, Abcam, Hong Kong; Anti-β-actin antibody, A2228, 1:1000, Sigma, St. Louis, MO) and were then exposed to the corresponding secondary antibody [Anti-rabbit or -mouse IgG (H+L) antibody, 7074S, 7076S, Cell Signaling Technology, Danvers, MA]. Protein bands were detected with an enhanced chemiluminescent substrate (EZ-ECL, Biological Industries, Kibbutz Beit-Haemek, Israel), scanned, and quantified by a Bio-imaging analyzer (Bio-Rad).

**Immunofluorescence**

To detect the localization of β-catenin and VEGF expression, double immunofluorescent staining of β-catenin with vWF and with VEGF were performed on aorta cross sections. The sections (2 cm below the renal artery) were then deparaffinized and rehydrated. After the antigen retrieval, the sections were incubated with 10% normal goat serum for 30 min at room temperature. Primary antibodies (Anti-vWF antibody, ab68545, 1:100, Abcam, Hong Kong; Anti-β-catenin antibody, ab23671, 1:100, Abcam, Hong Kong; Anti-VEGF antibody, NB100-648, 1:100, Novus Biologicals, Cambridge, United Kingdom) were applied at 4°C overnight and then detected with Dako REALTM EnVision+/HRP Rabbit/Mouse and 3,3-diaminobenzidine tetra-hydrochloride as chromogens (Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse, K5007, Dako Corporation, Carpinteria, California, USA). The sections were incubated with an unspecific isotype antibody to serve as negative controls. All sections were counterstained with hematoxylin. The vWF positively stained vessels in the adventitia were manually counted when the vessel was observed in an adjacent section, and the density was determined by Image-Pro Plus 5.1 (Media Cybernetics).
These pigs were then randomized into two groups, AS and AS + Endostar. The animals in the AS group received a single dose of saline, while those in the AS + Endostar group received an injection of Endostar. Both groups were placed on a hypercholesterolemic diet for an additional six weeks. In order to assess the atherosclerotic lesion in the AS group, one pig was sacrificed from the N and AS groups, respectively, at 12 weeks. The lesion was histopathologically evaluated as a characteristic feature of AS. Compared with the normal artery in the N group, a marked neointimal formation was observed and the intima–media thickness (IMT) was much greater in the AS group, indicating that the development of AS had been successfully induced by combining balloon injury with a hypercholesterolemic diet (Fig. 1).

**Weight Gain and Blood Analysis**

After the demonstration of a successful AS at 12 weeks, pigs in the N group were fed the normal diet for an additional six weeks. The remaining animals in the AS and AS + Endostar groups received a single dose of saline and Endostar, respectively, and were fed a hypercholesterolemic diet for another six weeks. At 18 weeks, all pigs were weighed, euthanized, and necropsied for additional analyses. The body weight and serum lipid levels, including total cholesterol, HDL, and LDL were markedly higher in the AS group than in the N group (Table 1), confirming the efficacy of the hypercholesterolemic diet. However, the AS +
Endostar group had values similar to those observed in the AS group (Table 1). Furthermore, levels of the serum inflammatory markers TNF-alpha, IL-6, and hs-CRP were higher in the AS group than in the N group but were similar in the Endostar group (Table 1), suggesting that the local inhibition of angiogenesis was not accompanied by a change in serum inflammatory markers.

**IMT as a Biomarker of AS**

It has been shown that IMT can be used as a biomarker to assess the progression and severity of AS in different animal models. Therefore, we measured IMT of the abdominal aorta in pigs in the three groups (Fig. 2A). IMT was 1.67-fold greater in the AS group than that in the N group, indicating that the porcine AS model was successfully established (Fig. 2B). Moreover, the Endostar treatment (AS + Endostar group) markedly inhibited IMT by 30.43% compared with the inhibition in the AS group (Fig. 2B).

**Vasa Vasorum Neovascularization in the Porcine AS Model**

A vascular marker, vWF, was present in the abdominal aorta among the three groups (Fig. 3A). The quantified vasa vasorum density revealed that it was much greater in pigs in the AS group (Fig. 3B). These findings demonstrated that neovascularization was a key feature of early AS and could be induced by the combination of balloon injury and a hypercholesterolemic diet. Moreover, the vasa vasorum density was markedly reduced by the Endostar treatment (AS + Endostar group), with levels similar to those in the N group (Fig. 3B).

**Local Expression of VEGF, β-Catenin, and TNF-Alpha in the Porcine AS Model**

VEGF is one of the essential regulators of angiogenesis and microvascular permeability, and β-catenin is a key intracellular signal transducer in the Wnt/β-catenin pathway, which plays an important role in the physiological and pathological of angiogenesis. Inflammation plays an important role in AS, and TNF-alpha is one of the most potent pro-inflammatory cytokines, which is actively involved in the progression and complications of AS. Western blotting revealed that the expression of VEGF, β-catenin, and TNF-alpha considerably increased in the atherosclerotic abdominal aorta in the AS group compared that in the N group (Fig. 4A). Quantitative analysis demonstrated a 3.03-, 1.66-, and 3.5-fold increase in β-catenin, VEGF, and TNF-alpha, respectively (Fig. 4B). More importantly, the Endostar treatment (AS + Endostar group) inhibited the expression of β-catenin, VEGF, and TNF-alpha in the atherosclerotic abdominal aorta compared with that observed in the AS group (Fig. 4A). Quantitative analysis demonstrated a 3.93-, 2.17-, and 1.71-fold decrease in the expression of β-catenin, VEGF, and TNF-alpha, respectively (Fig. 4B). Immunohistochemistry also revealed an increased immunoreactivity for VEGF (Fig. 5A, upper panel) and β-catenin (Fig. 5A, lower panel) in the AS group compared with those in the N and AS + Endostar groups. Furthermore, VEGF and β-catenin were found to be predominately localized in endothelial cells of the adventitial vasa vasorum in the abdominal
rum neovascularization, vessel wall inflammation, and the progression of AS in this model. The inhibitory effect of Endostar on vasa vasorum neovascularization was associated with a decrease in the expression of VEGF and β-catenin in the atherosclerotic abdominal aorta.

Vasa vasorum supply the outer walls of the larger arteries in the absence of diseases and are observed in systemic arteries, including the aorta, coronaries, carotids, and the femoral arteries. Experimental and clinical studies revealed that the hyperplasia of the adventitial vasa vasorum occurs in the early phases of

Discussion

In this study, we demonstrated that the combination of a hypercholesterolemic diet with balloon injury resulted in early AS in a swine model. Endostar, a potent angiogenesis inhibitor, attenuated vasa vasorum neovascularization, vessel wall inflammation, and the progression of AS in this model. The inhibitory effect of Endostar on vasa vasorum neovascularization was associated with a decrease in the expression of VEGF and β-catenin in the atherosclerotic abdominal aorta.

Fig. 2. IMT in different groups by histological analysis.

(A) Hematoxylin–eosin- and Verhoeff’s Van Gieson elastin-stained cross sections of the abdominal aorta. (B) Quantitation of IMT. *Significant difference compared with the control N group (p < 0.05); †Significant difference compared with the AS + Endostar group (p < 0.05). The Endostar treatment (AS + Endostar group) markedly inhibited IMT compared with the inhibition in the AS group. Scale bars: 500 μm.
Fig. 3. Immunohistochemistry of vWF in the abdominal aorta.

(A) Representative micrographs (40× magnification) and higher magnifications (100× and 400× magnification, respectively) of the inset region showed multiple vasa vasorum (arrows in A), which was stained brown with an anti-vWF antibody. Positive staining of the endothelium on the lumen served as an internal control. (B) Quantitation of vasa vasorum density in adventitia. * Significant difference compared with the N group (p < 0.05); # Significant difference compared with the AS + Endostar group (p < 0.05). The Endostar treatment (AS + Endostar group) significantly inhibited vasa vasorum density compared with the AS group. Scale bars: 500 μm (A, top panel), 200 μm (A, mid panel), and 50 μm (A, bottom panel).
of neovascularization attenuates the development of AS, suggesting it as an approach for preventing the disease\textsuperscript{14, 20, 32).

Endostar is a broad-spectrum angiogenesis inhibitor recommended by SFDA in 2005 for the treatment of non-small-cell lung cancer\textsuperscript{10, 14}). It was also a specific angiogenesis inhibitor that suppresses pathological angiogenesis and has no activity against wound healing. In our previous study, we found that Endostar could inhibit neovascularization and plaque growth in the rabbit model of AS\textsuperscript{23}). However, vasa vasorum in rabbits or mice is thinner than in porcine and human arteries\textsuperscript{37).} In this swine model of AS, we found that the density of vasa vasorum and IMT in the AS\textsuperscript{*} Endostar group was markedly less than that in the AS group. These findings indicated that Endostar also prevents neovascularization and neointima formation in the swine AS model.

Previous studies have shown the important role of vasa vasorum neovascularization as entry portals for leukocytes in the pathogenesis of AS\textsuperscript{8, 38).} The inhibition of vasa vasorum neovascularization reduces macrophage accumulation and progression of advanced AS\textsuperscript{39).} It has also been shown that an endostatin treatment reduces neovascularization in some inflammatory diseases as well as reduces infiltration with macrophages and lymphocytes\textsuperscript{40, 41).} Although the present study does not show the inflammatory cells in atherosclerotic plaque, it can be considered that the anti-atherosclerotic effect of Endostar contributes to the reduction of the number of entry portals for leukocytes into the atherosclerotic lesions.

Inflammation plays an important role in AS, and the atherosclerotic process when the tunica intima thickens up to 500 \(\mu\)m or even earlier, and vasa vasorum neovascularization in atherosclerotic plaque can be found in advanced lesions, particularly in vulnerable and disrupted plaques\textsuperscript{9, 14).} Furthermore, Hermann \textit{et al.} have demonstrated that vasa vasorum neovascularization occurred before the development of endothelial dysfunction of the host vessel\textsuperscript{31).} Therefore, vasa vasorum neovascularization was considered as an important marker of AS. In this porcine model of early AS, our results showed an increased density of the adventitial vasa vasorum neovascularization distributed in excess of 500 to 800 \(\mu\)m from the lumen, and little vasa vasorum neovascularization in atherosclerotic plaque can be found, which is similar with the distribution in human early atherosclerotic plaque\textsuperscript{30).}

Vasa vasorum neovascularization plays a pivotal role in the pathogenesis of human AS\textsuperscript{30, 32).} Previous studies have demonstrated increased E-selectin, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and lectin-type oxidized LDL receptor 1 levels in the endothelium of vasa vasorum compared with those on the artery surface\textsuperscript{11, 33).} Furthermore, the structural integrity of plaque neovascularization was incomplete in AS\textsuperscript{14).} Thus, vasa vasorum neovascularization was considered as the major entry portals for leukocytes and lipids into the atherosclerotic lesions, which accelerates the progression and destabilization of AS plaque\textsuperscript{35, 36). These observations suggest that vasa vasorum neovascularization, inflammation, and lipids were potential therapeutic targets to prevent AS. Several studies have demonstrated that the inhibition of neovascularization attenuates the development of AS, suggesting it as an approach for preventing the disease\textsuperscript{14, 20, 32).}

Endostar is a broad-spectrum angiogenesis inhibitor recommended by SFDA in 2005 for the treatment of non-small-cell lung cancer\textsuperscript{10, 14).} It was also a specific angiogenesis inhibitor that suppresses pathological angiogenesis and has no activity against wound healing. In our previous study, we found that Endostar could inhibit neovascularization and plaque growth in the rabbit model of AS\textsuperscript{23).} However, vasa vasorum in rabbits or mice is thinner than in porcine and human arteries\textsuperscript{37).} In this swine model of AS, we found that the density of vasa vasorum and IMT in the AS\textsuperscript{*} Endostar group was markedly less than that in the AS group. These findings indicated that Endostar also prevents neovascularization and neointima formation in the swine AS model.
TNF-alpha is one of the most potent pro-inflammatory cytokines. It has been identified that TNF-alpha is actively involved in the progression and complications of AS and that inhibition of tumor necrosis factor-alpha reduces AS both in human diseases and animal models. Our results showed that the
expression of TNF-alpha increased in the AS group and markedly decreased by the Endostar treatment, suggesting that Endostar reduces vessel wall inflammation in the swine AS model. Systemic inflammatory markers, such as TNF-alpha, hs-CRP, and IL-6 are elevated in patients with CAD. In our study, serum levels of TNF-alpha, hs-CRP, and IL-6 were markedly higher in the AS group than in the N group but showed no considerable difference after the Endostar treatment. These results suggest that the local inhibition of angiogenesis was not accompanied by a change in serum inflammatory markers. Altogether, we found that the Endostar treatment inhibited the local TNF-alpha production but did not change the systemic level of TNF-alpha. Although our study design did not show any direct causality, these results indicated that the inhibitive effect of Endostar on local TNF-alpha expression is because of the inhibition of vasa vasorum neovascularization. These findings can further support the idea of the inhibition of vasa vasorum neovascularization as a potential therapeutic strategy for the prevention of plaque development and destabilization.

VEGF is one of the most important regulators of angiogenesis and microvascular permeability. It has been shown that VEGF enhances atherosclerotic plaque progression by increasing the endothelial surface for leukocytes and lipids into the vessel wall. Moreover, previous studies demonstrated that VEGF is closely associated with inflammatory processes in inflammatory diseases (i.e., AS, rheumatoid arthritis, inflammatory bowel disease, and osteoarthritis). It has been shown that VEGF induces plaque expansion in ApoE knock-out mice by promoting de novo leukocyte recruitment. We found that compared with the N group, the expression of VEGF in the AS group markedly increased, which is consistent with the previous study on human atherosclerotic plaques. The Endostar treatment resulted in a marked decrease in VEGF expression. Using immunohistochemical analyses, we found an increased immunoreactivity for VEGF in the AS group compared with that in the N and AS + Endostar groups. Furthermore, VEGF predominately localized with endothelial cells of the adventitial vasa vasorum in the abdominal aorta. These results indicate that the inhibitive effect of Endostar on vasa vasorum neovascularization and vessel wall inflammation is because of the suppression of VEGF production in the atherosclerotic model group in addition to the presence of less VEGF-producing vasa vasorum.

β-catenin is a protein involved in cell–cell adhesion and also acts as a key intracellular signal transducer in the Wnt/β-catenin pathway. Previous studies have demonstrated that Wnt/β-catenin pathway plays an important role in the physiological and pathological of angiogenesis. In our previous study, we found that Endostar suppresses angiogenesis through the inhibition of the Wnt/β-catenin pathway. In this study, we found that the amount of β-catenin was markedly higher in the atherosclerotic abdominal aorta. Immunohistochemistry staining demonstrated a strong immunoreactivity for β-catenin in the AS group compared with that in the N and AS + Endostar groups. Further, the presence of β-catenin mainly localized with endothelial cells of adventitial vasa vasorum in the abdominal aorta. The Endostar treatment resulted in a marked reduction of β-catenin expression compared with that in the AS group. These data suggest that the Wnt/β-catenin pathway has a possible role in vasa vasorum neovascularization, and the inhibitory effect of Endostar on vasa vasorum neovascularization was partly through the Wnt/β-catenin pathway. There are several limitations to the study. First, the direct visualization of vasa vasorum neovascularization using contrast-enhanced ultrasound and magnetic resonance has recently emerged for the detection of AS. This study was not designed to monitor antiangiogenic interventions on vasa vasorum neovascularization within the vessel wall using these novel imaging techniques. Therefore, it is necessary to improve it in our future study. Second, previous studies have shown the important role of vasa vasorum neovascularization as entry portals for macrophage infiltration in the pathogenesis of AS. Therefore, it is necessary to assess the amount of macrophage infiltration in the swine AS model. However, because of the lack of effective antibody, macrophage infiltration was not recorded in our study. Finally, although Endostar was well tolerated, the reported side effects of antiangiogenic drugs, including thrombosis, need to be avoided. A recent study has shown that the combination of cardiac magnetic resonance molecular imaging with alpha(v)beta3 integrin-targeted fumagillin nanoparticles can not only inhibit angiogenesis but also monitor neovascularization in AS. The concept of selective molecular targeting for anti-neovascularization may minimize the risk of thrombosis.

In conclusion, the present study demonstrated that the Endostar treatment inhibited the expression of VEGF, β-catenin, and TNF-alpha and vasa vasorum neovascularization in the atherosclerotic abdominal aorta, attenuating the development of AS, as induced by hypercholesterolemic diet and balloon injury surgery in pigs. Therefore, this study supports a role of anti-neovascularization as a potential therapeu-
tic strategy for preventing AS.

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Abbreviations

atherosclerosis, AS; intima-media thickness, IMT; coronary artery disease, CAD; State Food and Drug Administration of China, SFDA; vascular endothelial growth factor, VEGF; von Willebrand factor, vWF; tumor necrosis factor-alpha, TNF-alpha; high-sensitivity C-reactive protein, hs-CRP; interleukin-6, IL-6; high density lipoprotein, HDL; low density lipoprotein, LDL; high sensitivity growth factor, VEGF; von Willebrand factor, vWF; vascular endothelial growth factor, VEGF; tumor necrosis factor-alpha, TNF-alpha; high-sensitivity C-reactive protein, hs-CRP; interleukin-6, IL-6; low density lipoprotein, LDL; high density lipoprotein, HDL

Conflict of Interests

The authors declare that they have no conflict of interests.

References

4) Zhang C: The role of inflammatory cytokines in endothelial dysfunction. Basic Res Cardiol, 2008; 103: 398-406
21) Wang TB, Wei XQ, Lin WH, Shi HP, Dong WG: The


52) Dejana E: The role of Wnt signaling in physiological and pathological angiogenesis. Circ Res, 2010; 107: 943-952