ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Feb; 22 (2): 117 - 120
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VEGF induced hyperpermeability in bovine aortic endothelial cell and inhibitory effect of salvianolic acid B[1]

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KEY WORDS vascular endothelial; growth substances; permeability; salvianolic acid B

ABSTRACT

AIM: To examine the effect of recombinant human vascular endothelial growth factor (VEGF) on the low density lipoprotein (LDL) permeability of bovine aortic endothelial cells (BAEC) and the inhibitory effect of salvianolic acid B *in vitro*. **METHODS**: The confluence BAEC monolayers were cultured with normal medium and with medium containing VEGF or salvianolic acid B at various concentrations and for various time periods. The iodine labeled LDL flux across the monolayers was then performed, and the radioactivity was measured by SN-695 automatic liquid scintillation counter. **RESULTS**: Addition of purified human recombinant VEGF to the BAEC monolayers could significantly increase the permeability of the monolayer to ^{125}I-LDL ($P<0.01$). The permeability-increasing activity of VEGF on the BAEC monolayers was both dose and time dependent. Salvianolic acid B could markedly inhibit the VEGF-induced hyperpermeability in BAECs ($P<0.01$). **CONCLUSION**: VEGF plays a role in the formation and development of atherosclerosis, and salvianolic acid B has inhibitory effect on VEGF-induced hyperpermeability in BAEC.

INTRODUCTION

The vascular permeability factor (VPF) was originally described as a tumor secreted protein which caused vascular leakage. This protein was later independently cloned and named vascular endothelial growth factor (VEGF)[1]. Alternative splicing of VEGF mRNA accounts for 4 isoforms of 121, 165, 189, and 206 amino acids, of which the 165aa form is most abundant *in vivo*. The protein is a homodiner of M_r 34 000 - 45 000. As suggested by its name, VEGF/VPF is an important regulator of vasculogenensis, angiogenesis, and vascular permeability *in vivo*.

Low density lipoprotein (LDL) and oxidative modified low density lipoprotein (Ox-LDL) appear to play a central role in the development of atherosclerosis. It seems important therefore to understand factors determining the accumulation of LDL in the arterial wall initma. VEGF is animportant regulator of vascular permeability, but it induced hyperpermeability of LDL has not been reported. The hypothesis that VEGF can increase LDL permeability in aoritic endothelium, particularly in relation to the risk of development of atherosclerosis remains to be investigated.

To gain a better understanding of the biological effects of VEGF, especially the relationship between the VEGF-induced hyperpermeability on endothelial cells and atherosclerosis, experiments were conducted to determine the effect of VEGF on the permeability of cultured bovine aortic endothelial cells (BAEC).

Salvianolic acid B, one of the effective pure compounds of total salvianolic acid, was extracted from Salvia miltorrhiza solution. It has been reported that salvianolic acid B possesses strong antioxidant action both *in vivo* and *in vitro*, and has certain protective effects on cardiovascular system[2]. In this paper, we concentrate on the regulation of VEGF to increase the ^{125}I-LDL permeability of the BAEC monolayers and the action of salvianolic acid B *in vitro*.

MATERIALS AND METHODS

Reagents Recombinant human VEGF was obtained from CalBiochem (San Diego, CA), LDL was

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Received 2000-06-19 Accepted 2000-10-27

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Feb; 22 (2)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

purchased from Sigma Co (USA). ^{125}I-LDL was labeled by Institute of Atomic Nucleus (Chinese Academy of Medical Sciences, Shanghai). Salvianolic acid B was kindly provided by Dr ZHANG Wei-Dong (Second Medical Military University, Shanghai). Tanswell System cell culture dishes were obtained from Costar Co (6.5 mm diameter, USA).

Cell culture Bovine aortic endothelial cells (BAEC) were harvested as previously described[3] and cultured in minimum essential medium (MEM) with 10 % fetal bovine serum, gentamicin 50 mg/L. Permeability studies performed were done using confluent BAEC monolayers in their third to sixth passages.

The BAEC were cultured in a Transwell system. The Transwell system was composed of two compartments, a removable Transwell insert (upper or apical chamber) and a bottom well (lower or basolateral chamber). The two compartments were separated by a microporous polycarbonate membrane (0.1 μm pore size) that covered the bottom of insert and permitted exchange of medium between these two chambers. The BAEC were seeded at 5×10^8/L onto the Transwell inserts. The Transwell insert membranes had been pretreated with collagen 2 g/L prior to seeding. The insert chamber contained 100 μL BAEC medium and the bottom chamber contained 600 μL MEM. The cells were grown in a 37 ℃ incubator with 5 % CO_2 and 95 % humidity. The cultured medium was changed on the third day after plating and every other day thereafter. The experiments were conducted when BAEC formed tight monolayers.

Characterization of effect of VEGF on BAEC monolayers To examine the effect of VEGF on the BAEC, the confluent BAEC monolayers were incubated with culture medium containing recombinant human VEGF at various concentrations and for various time periods. The transendothelial LDL flux across the monolayers was then performed[4].

Transendothelial flux measurement (permeability assay) After the establishment of a confluent BAEC monolayer, the flux of ^{125}I-LDL across the filter grown BAEC monolayers was measured. MEM (100 μL) containing ^{125}I-LDL was added to the existing apical medium (100 μL) of each apical chamber (donor chamber) to give a final ^{125}I-LDL concentration of 3.7 MBq/L. The appearance of ^{125}I-LDL in the basolateral chamber was measured by removing the basolateral chamber medium at various times, and the radioactivity in the sample was determined using SN-965 automatic liquid scintillation counter. For comparison, ^{125}I-LDL flux across filters alone and collagen coated filters was also examined. All experiments were carried out at 37 ℃. The amount of radiolabeled tracer that penetrated the BAEC monolayers was expressed as follows: penetrated tracer (%) = $C_r/C_d \cdot 100$, where C_r is the count of the radiotracer appearing in the receiver chamber, C_d is the count of initial tracer in the donor chamber.

Characterization of the inhibitory effect of salvianolic acid B To examine the inhibitory effect of salvianolic acid B on VEGF-induced hyperpereability in BAEC, the confluent BAEC monolayers were co-incubated with culture medium containing VEGF 100 μg/L (100 μL) and salvianolic acid B (100 μL) at various concentrations for 1 d. All experiments were carried out in triplicate and were repeated at least once.

Statistical analysis Results were presented as $\bar{x} \pm s$. Statistical analysis was performed with ANOVA.

RESULTS

Establishment of BAEC monolayers in the Transwell system BAEC were cultured on collagen-coated microporous membranes in Transwell as described in METHODS. The BAEC reached confluence on 4, and formed a tight monolayer approximately 4 or 5 after plating.

Characterization of effect of VEGF on the permeability of BAEC To determine the time dependent effect of VEGF, we incubated confluent BAEC monolayers with medium containing VEGF (100 μg/L). Fig 1 shows the various time period ^{125}I-LDL permeability results from BAEC monolayers treated with VEGF for 1 d. A significant increase in the permeability of VEGF-treated BAEC was observed at 30 min compared with that of the control monolayers. Twenty-nine percent of ^{125}I-LDL penetrated the BAEC monolayers, whereas only twenty percent of ^{125}I-LDL moved across control monolayers at 60 min.

We also examined the dose-dependent effect of VEGF on the permeability of the BAEC monolayers by incubating various amounts of VEGF with the monolayers as described above for 1 d (Fig 2). Results showed that the VEGF concentrations of 50 μg/L or greater were required to stimulate significant increase in the permeability of the monolayers. The permeability-increasing activity of VEGF reached a plateau at about 100 μg/L.

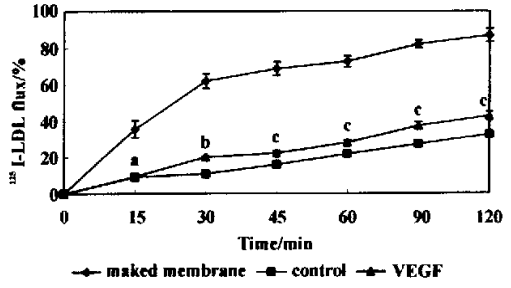

Fig 1. Time-dependent effect of VEGF on the permeability of BAEC monlayers. $\bar{x} \pm s$. $n = 6$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

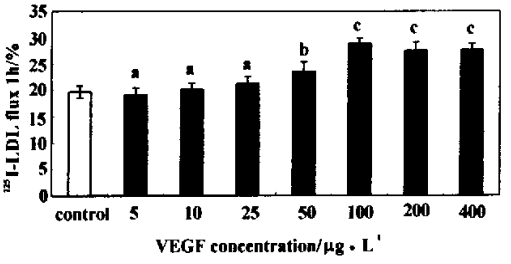

Fig 2. Dose-dependent effect of VEGF on permeability of BAEC monlayers. $\bar{x} \pm s$. $n = 6$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control group.

The inhibitory effect of salvianolic acid B

We observed the action of various concentrations of salvianolic acid B on VEGF-treated BAEC. We found that the salvianolic acid B at the concentration from 135 – 1.35 nmol/L could strongly inhibit the hyperpermeability induced by VEGF (100 μg/L) ($P < 0.01$), at the concentration of 0.135 nmol/L, it also had significant inhibitory effect compared with the control groups ($P < 0.05$). At the range of 135 – 0.135 nmol/L, it expressed inhibition a dose-dependent manner (Tab 1).

DISCUSSION

The clinical manifestations of atherosclerosis, coronary heart diseases, and peripheral vascular disease are the major causes of morbidity and mortality in the developed countries. The accumulation of atherogenic lipoproteins in the arterial wall intima constitutes a fundamental event in atherosclerosis[5]. LDL is the most abundant atherogenic lipoprotein in plasma and high plasma levels of LDL are related to the development of atherosclerosis[6]. It seems important therefore to understand factors determining transport of LDL into the arterial wall. The aim of our study was to observe the VEGF-induced LDL hyperpermeability on cultured BAEC, particularly in relation to the risk of developing atherosclerosis.

Tab 1. Inhibitory effect of salvianolic acid B on VEGF-induced hyperpermeability in BAECs. $\bar{x} \pm s$. $n = 6$. $^bP < 0.05$, $^cP < 0.01$ *vs* control (VEGF).

Groups	^{125}I-LDL flux 1 h/%
Medium(BAEC)	20.1 ± 1.0
Control(VEGF)	29.6 ± 1.0
Antibody of VEGF	21.1 ± 1.5^c
Salvianolic acid B/nmol·L^{-1}	
135	21.8 ± 0.9^c
13.5	23.3 ± 0.8^c
1.35	25.5 ± 0.6^c
0.135	26.4 ± 1.0^b
0.0135	29.6 ± 0.9

Endothelial cells are not only a simple physiological barrier, but also an active metabolic pool. It is generally agreed that the vascular endothelium plays a key role in the initiation of the atherosclerotic processes. Furthermore, the functional and structural changes of endothelium are associated with the occurrence and developing of cardiovascular diseases. The endothelial injury hypothesis suggests that it is responsible for the initiation of atherosclerotic disease processes[7] and LDL is a major factor inducing endothelial function disturbance. The endothelial injury leads to significant increase in LDL premeability. VEGF, a vascular endothelium specific cytokine, stimulates vascular endothelial cell growth *in vitro*, induces angiogensis, and enhances vascular permeability *in vivo*. Though the effect of VEGF on the endothelial growth and angiogenesis is undergoing intensive study *in vitro* and *in vivo* at the physiological, biochemical and molecular levels, the studies on the permeability-enhancing activity of VEGF are rare[8,9]. In this study, we investigated the effect of VEGF on the LDL permeability of cultured BAEC *in vitro*, we found that addition of purified VEGF to the BAEC monolayers markedly increased the permeability of the monolayers to ^{125}I-LDL (~ 50 %). The permeability-increasing activity of VEGF on the BAEC monolayers is both dose and time dependent.

Salvia miltiorrhiza Bunge is a Chinese herb widely used for the treatment of atherosclerosis-related disorder.

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Feb; 22 (2)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

Salvianolic acid B, a water-soluble polypgennolic antioxidant isolated from the roots of this plant, was found to scavenge 1,1-diphenyl-2-picryldrazyl radicals and inhibit LDL oxidation more effectively than probucol[10]. Previous studies had demonstrated that salvianolic acid B could significantly scavenge oxygen free radicals[11], prevent endothelial damage[10] and decrease the intracellular calcium concentration[11]. In present studies, we found that salvianolic acid B could dose-dependently inhibit the permeability-increasing activity of VEGF on the BAEC monolayers in 135 - 0.135 nmol/L. The mechanism of this action will be investigated in further studies.

To summarize, current studies demonstrate that VEGF is able to induce a permeability increase in cultured BAEC. The present data suggest that VEGF may play an important role in regulating the LDL permeability and salvianolic acid B can inhibit the permeability-increasing effect of VEGF.

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VEGF 诱导牛主动脉内皮细胞通透性升高及丹酚酸 B 对其的抑制作用[1]

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关键词 血管内皮; 生长物质; 通透性; 丹酚酸 B

目的: 考察血管内皮生长因子/血管通透因子 (VEGF) 对体外培养牛主动脉内皮细胞脂蛋白通透性的影响及丹酚酸 B 的抑制作用. **方法**: 将不同浓度的 VEGF、丹酚酸 B 及培养基加入牛主动脉内皮细胞单层, 与 ^{125}I - LDL 共孵育, 在不同的时间段用 SN-695 型液闪计数器测其通过细胞单层的百分率. **结果**: VEGF 可显著增强牛主动脉内皮细胞单层对 ^{125}I-LDL 的通透性 ($P < 0.01$), 这种增加具有时间和剂量依赖性. 丹酚酸 B 能显著抑制 VEGF 诱导的这种高通透 ($P < 0.01$). **结论**: 上述结果提示 VEGF 在动脉粥样硬化的形成和发展过程中起一定作用. 丹酚酸 B 对 VEGF 诱导血管内皮通透性升高有显著的抑制作用.

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