ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Apr; 22 (4): 335-339
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Effect of lysophosphatidylcholine on expression of vascular endothelial growth factor in ECV304 cells[1]

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KEY WORDS lysophosphatidylcholines; vascular endotheliun; growth substances; enzyme-linked immunosorbent assay; immunohistochemistry

ABSTRACT

AIM: To determine the effect of lysophosphatidylcholine (LPC) on the expression of vascular endothelial growth factor (VEGF) in human umbilical veins endothelial cell line (ECV304). **METHODS**: VEGF protein production from ECV304 cells was determined by enzyme-linked immunosorbent assay (ELISA). VEGF protein and its receptor were also examined by immunohistochemical staining. After culturing for 24 h with LPC, VEGF mRNA expression in ECV304 was examined by *in situ* hybridization. **RESULTS**: Cultured ECV304 cells could express VEGF receptor and a strong expression of VEGF receptor was induced by LPC 5 mg/L. LPC upregulated VEGF protein secreted from the ECV304 cells in a time- and concentration-dependent manner and LPC also stimulated VEGF mRNA expression in the ECV304 cells. **CONCLUSION**: LPC could induce a strong expression of VEGF in ECV304 cells.

INTRODUCTION

During the process of atherosclerosis (AS), a number of growth regulatory molecules and cytokines, such as VEGF, platelet-derived growth factor (PDGF), tumour necrosis factor (TNF), and interleukin-1 (IL-1) may be formed and released from endothelial cells (EC), smooth muscle cells (SMC), and macrophage within the lesions[1]. These growth factors and cytokines seem to play an important role in the progression of AS.

Recent findings indicate that oxidised low density lipoprotein (ox-LDL) may play a key role in the atherosclerotic process[2,3]. Immunohistochemistry studies suggest that ox-LDL accumulates in atherosclerotic lesions[4-6] and ox-LDL can modulate growth factor or cytokine production from ECs, SMCs, or macrophages[7-9]. Thus, ox-LDL can act as a mediator of many cellular events relevant to atherogenesis. LPC, which is increased in the plasma of hypercholesterolemic patients, is a component of ox-LDL, and as such, may play an important role in AS[10,11].

VEGF/VPF is a heparin-binding, homodimeric glycoprotein of M_r 46 000 belong to the PDGF family[12]. VEGF is an important special mitogen and chemoattractant for endothelial cells both *in vitro* and *in vivo*[13] as well as a potent enhancer of vascular permeability[14]. VEGF exists in four different isoforms: VEGF121, VEGF165, VEGF189, and VEGF206[15]. It exerts its biological functions through two high-affinity tyrosine kinase receptors expressed predominantly by endothelial cells, the kinase insert domain-containing receptor (KDR) and the fms-like tyrosine kinase-1 receptor (Flt-1). The murine homologue of KDR is also known as fetal liver kinase-1 receptor (Flk-1)[16].

The aim of the study was to understand the interaction between LPC and VEGF, the effects of LPC on VEGF protein and VEGF mRNA expression in ECV304 cells, a human umbilical veins endothelial cell line, on stimulation by LPC.

MATERIALS AND METHODS

Cell culture Human umbilical vein endothelial cell line ECV304 was obtained from Kenen Company, Beijing. The ECV304 cells were cultured in modified Eagle's medium (MEM; Gibco) containing 20 % fetal bovine serum (FBS; Gibco). In the experiments, ECV304 cells at confluence were pretreated with serum-free MEM for 24 h. The medium was then replaced with fresh, serum-free medium containing LPC (Sigma) incu-

[1]Project supported by the National Natural Science Foundation of China, No 39870870.
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Received 2000-07-03 Accepted 2001-01-19

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Apr; 22 (4)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

bated for the indicated periods of time.

VEGF protein quantification The ECV304 cells were stimulated with LPC 2.5 mg/L for 2 h to 24 h or with varying doses of LPC (1, 2.5, 5, 7.5, and 10 mg/L) for 24 h at 37 ℃ in a 5 % CO_2 and 95 % ambient air environment. After incubation of the ECV304 cells as indicated, the conditioned medium was collected and centrifuged at 1000 × *g* for 5 min, and the supernatant was used for the measurement of VEGF protein with a Quantikine Human VEGF ELISA kit (Cytimmune Sciences INC). The absorbance (*A*) of VEGF suandards (0.2, 0.8, 3.1, 12.5, 50, 200 μg/L) was detected from the kit and the standard curve was obtained according to its corresponding *A*.

Immunohistochemistry Slips were placed into 35 millimeter culture dishes (Nalge Nunc International) and the ECV304 cells were added into the dishes. When the cells reached confluence, they were incubated with serum-free MEM for 24 h to eliminate the effect of serum. The cells were stimulated by LPC 5, 10, 20 mg/L at 37 ℃ for 6 h, then the slips were washed with phosphate-buffer saline (PBS PH7.2) 3 times. The sections were immersed in 4 % paraformaldehyde for 15 min and washed with PBS[17]. The sections were incubated with human VEGF antibody (4 mg/L) or human VEGF receptor antibody (Santa Cruz) overnight at 4 ℃ and washed with PBS. The sections were incubated for 30 min with biotinylated secondary antibody (SABC). After being washed with PBS, the AB enzyme reagent (DAKO) was added for 30 min and washed with PBS. The peroxidase substrate solution containing 3, 3′-diaminobenzidine (DAB) was applied. The sections were counterstained in Gill's formulation # 2 hematoxylin (Sigma) for 5 s and immediately washed with several changes of H_2O. After dehydrating the sections, 2 drops of permanent mounting medium was added and the sections were covered with a glass coverslip.

***In situ* hybridization analysis** Slips were placed into 35 mm culture dishes (Nalge Nunc International) with the ECV304 cells. After incubating with serum-free MEM for 24 h, the cells were stimulated by LPC 5 mg/L at 37 ℃ for 24 h, in the control dish only MEM was added. The sections were immersed in 4 % paraformaldehyde for 5 min and washed with PBS. The sections were then subjected to *in situ* hybridization as detailed in Brown *et al*[18]. The VEGF cRNA probe was purchased from Santa Cruz. The probe was marked and detected with Digoxin detection kit (Boehringer Mannheim).

Statistical methods Each experiment was repeated at least three times. Where indicated, data were presented as $\bar{x} \pm s$. Differences were considered statistically significant when $P < 0.05$ as analyzed by paired *t*-test.

RESULTS

VEGF standard curve The standard curve was plotted on a semi-log graph paper. Known concentrations of VEGF were plotted against the corresponding *A* (Fig 1). The standard curve had a sigmoid shape that showed an inverse relationship between VEGF concentration and the corresponding *A*. The *A* of VEGF standards were detected from 0.2 to 200 μg/L by ELISA, and the equation of the VEGF standard curve was $y = -0.3336x + 2.2961$ ($R^2 = 0.9768$, $n = 3$) (Fig 1). The test samples of VEGF were calculated by the equation according to its corresponding *A*.

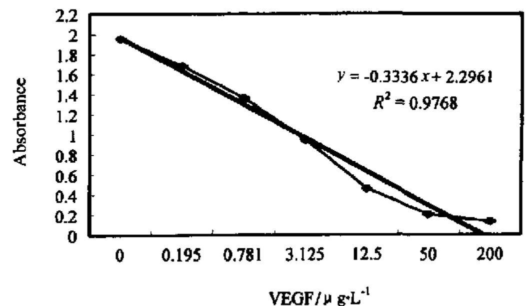

Fig 1. The standard curve of VEGF standards. $n = 4$. $\bar{x} \pm s$.

Effect of LPC on VEGF protein levels in a conditioned medium To examine whether LPC stimulates VEGF production by ECV304 cells, VEGF levels were determined in conditioned medium from cells that had been incubated with LPC 1 to 10 mg/L for 24 h by ELISA. VEGF production from ECV304 cells was increased by exposure to LPC in a concentration-dependent manner (Fig 2). A maximal concentration of VEGF protein was 2.5 μg/L ± 0.2 μg/L when ECV304 cells incubated with 2.5 mg/L LPC. Whereas, the control VEGF concentration was 0.4 μg/L ± 0.1 μg/L. ($P < 0.01$ *vs* control group, $n = 4$). As shown in Fig 3, ECV304 cells were incubated with LPC 2.5 mg/L for various times. LPC upregulated VEGF production from ECV304 cells in a time-dependent manner, and a maxi-

mal effect was observed at 24 h. The maximal VEGF concentration was 2.5 μg/L ± 0.2 μg/L. ($P < 0.01$ *vs* control group, $n = 4$).

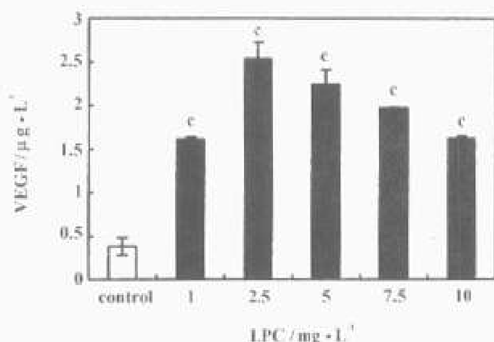

Fig 2. Concentration-dependent curve of VEGF protein production from ECV304 cells stimulated by LPC. $n = 4$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control group.

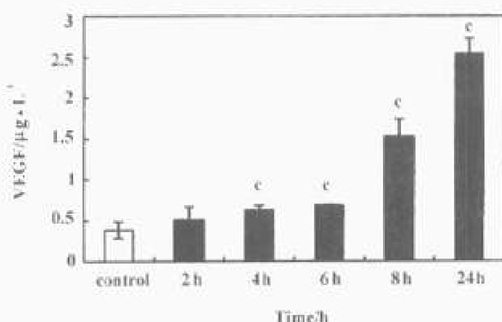

Fig 3. Time-dependent curve of VEGF protein production from ECV304 cells stimulated by LPC. $n = 4$. $x \pm s$. $^cP < 0.01$ *vs* control group.

Induction of VEGF and its receptor in ECV304 cells as visualized by immunohistochemical staining VEGF protein secreted from ECV304 cells, with or without LPC stimulation, showed negative immunoreactivity and a weak staining of VEGF receptor antibody was observed in cultured cells (photograph not shown). After the cells were exposed to LPC at concentrations between 5 and 20 mg/L for 6 h. LPC increased VEGF receptor expression in ECV304 cells, as shown in Fig 4. An intense staining of VEGF receptors was observed on stimulation with LPC 5 mg/L.

Induction of VEGF mRNA in ECV304 cells stimulated by LPC To examine whether LPC stimulates VEGF mRNA expression in ECV304 cells, VEGF mRNA were determined by *in situ* hybridization analysis. There was no staining of VEGF mRNA in cultured ECV304 cells, but there was strong staining after exposure to LPC 5 mg/L (Fig 5).

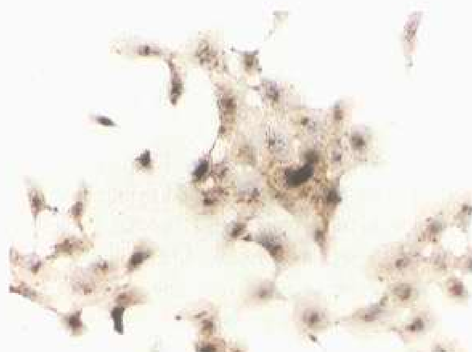

Fig 4. The photomicrograph of VEGF antibody counterstained with Gill's formulation # 2 hematoxylin after stimulation with LPC. Strong staining with VEGF receptor was observed after incubation with LPC 5 mg/L. × 400.

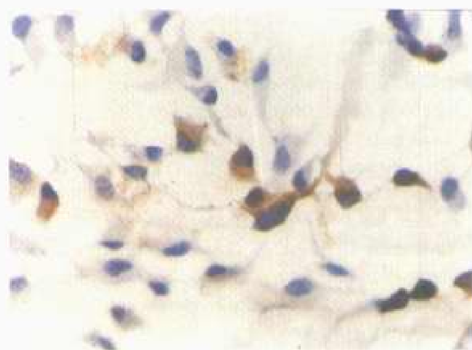

Fig 5. The Photomicrograph of VEGF mRNA after stimulation with LPC in *in situ* hybridization analysis. Strong staining with VEGF was observed after incubated with LPC 5 mg/L. × 400.

DISCUSSION

A distinct expression of VEGF and its receptors has been well established in atherosclerotic lesions in human coronary arteries[19], suggesting a pathophysiological significance of VEGF in progression of atherosclerosis. Ramos *et al*[6] demonstrated that early atherosclerotic lesions in human exhibited intense VEGF immunoreactivity in subendothelial macrophage-rich regions of the thickened intima. In atherosclerotic plaques, on immunohistochemical staining it VEGF mRNA staining was observed

in foam cell-rich regions adjacent to the lipid core or the neovascularized basal regions of plaque. Highpower-field observation revealed that VEGF was localized in the extracellular space.

We found the presence and distribution of VEGF and its receptors in ECV304 cells. In cultured ECV304, there was no per se staining for VEGF protein and VEGF mRNA. A weak staining for VEGF receptors antibody was observed after stimulation by LPC, VEGF staining was distinct on the cell surface and cytochylema. LPC was shown to upregulate VEGF mRNA expression in ECV304 cells and to stimulate VEGF protein secretion from the cells in a time- and concentration-dependent manner. The results suggested that LPC might increase the action of VEGF.

In summary, we have demonstrated that LPC, a component of ox-LDL, induced VEGF protein and VEGF mRNA expression in cultured ECV304 cells. Our findings suggest that interaction between LPC and VEGF may be involve a in the development of human atherosclerosis.

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ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Apr; 22 (4)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

溶血磷脂酰胆碱对 ECV304 细胞中血管内皮生长因子表达的影响[1]

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关键词 溶血磷脂酰胆碱类；血管内皮；生长物质；酶联免疫吸附测定；免疫组织化学

目的：研究溶血磷脂酰胆碱(LPC)对内皮细胞(EC)中血管内皮生长因子(VEGF)表达的影响. **方法**：在人脐静脉内皮细胞株 ECV304 培养基中加入不同浓度的 LPC，培养不同时间，用基础酶联免疫吸附实验(ELISA)检测各组 EC 条件培养基中 VEGF 蛋白含量；用免疫组织化学法检测 EC 中 VEGF 蛋白及其受体的表达；用原位杂交检测 VEGF 信使核糖核酸(VEGF mRNA)的表达. **结果**：培养的 ECV304 能表达 VEGF 受体，在胞浆内呈棕色颗粒，当 LPC 刺激后，阳性增强. 原位杂交结果显示，培养的 ECV304 中未见 VEGF mRNA 的表达，当 LPC 刺激后可见 VEGF mRNA 的高表达，在胞浆内呈棕色颗粒. ELISA 结果显示 LPC 可使 ECV304 条件培养基中 VEGF 蛋白含量明显增加，且具有时间和剂量依赖性. **结论**：LPC 能诱导 ECV304 表达高水平的 VEGF.

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