ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Apr; 20 (4): 313 - 318
http://www.simm.ac.cn; www.chinainfo.gov.cn/periodical

Intraventricular vascular endothelial growth factor antibody increases infarct volume following transient cerebral ischemia[1]

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KEY WORDS transient cerebral ischemia; reperfusion injury; vascular endothelium; endothelial growth factors; immunohistochemistry

ABSTRACT

AIM: To clarify the role of vascular endothelial growth factor (VEGF) in neuronal damage induced by cerebral ischemia. **METHODS**: Expression of VEGF in adult rat brain was measured by immunohistochemistry. Transient middle cerebral artery occlusion (MCAO) model was induced by placing a nylon thread in the lumen of the internal carotid artery. The infarct volume was shown with 2,3,5-triphenyltetrazolium chloride (TTC) staining and quantitated by computer image analyzer with and without VEGF antibody treatment. **RESULTS**: VEGF expression was widely distributed in neuronal cells besides vascular endothelial cells, and the neuronal distribution of VEGF was specific. After intraventricular treatment with VEGF antibody (0.1 g·L^{-1} daily, for 7 d following the ischemia), infarct volume in the antibody treatment was increased versus vehicle-treated rats [(21.6 ± 2.7 *vs* 16 ± 6) mm^3, $P < 0.05$] respectively. **CONCLUSION**: Intraventricular injection of VEGF antibody increased the infarct volume after focal cerebral ischemia in rats, suggesting that expression of neuronal VEGF may be one of neuronal protective mechanisms.

[1] Project supported by the National Natural Science Foundation of China (No 39730170) and by Shanghai Municipal Health Bureau (No 97BR022).
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Received 1998-06-29 Accepted 1998-10-16

INTRODUCTION

Vascular endothelial growth factor (VEGF) is a dimeric glycoprotein of approximately 46 kDa that is structurally related to platelet-derived growth factor[1]. Initially identified by its ability to increase vascular permeability, VEGF has also been shown to possess a potent angiogenic activity[2]. In addition to its tumor-associated angiogenesis and vascular permeability, VEGF has been demonstrated to express in the cultured astrocyte, cardiac myocytes, cornea, and ovarian follicles[3-5]. Moreover, VEGF mRNA levels were reversibly increased in cultured cells subjected to hypoxia[6], and the expression of VEGF in cardiac myocytes was induced by ischemia *in vitro* and *in vivo*[7,8]. However, the expression of VEGF mRNA in the cultured astrocytes or cardiac myocytes could be enhanced by hypoxic stimulation that was not directly related to the angiogenesis[4,7]. In our previous study, VEGF mRNA and VEGF were time-dependently expressed in the neuronal cells following the cerebral ischemia, suggesting that VEGF may participate in neuronal pathophysiological process after cerebral ischemia[9]. However, a comprehensive analysis of the distribution of VEGF within the CNS has not been undertaken, and the functional significance of the expression of neuronal VEGF induced by ischemia is not known. The present study was to observe the neuronal distribution of VEGF in central nervous system and to assess the role of VEGF in pathophysiologic process after cerebral ischemia.

MATERIALS AND METHODS

Immunohistochemistry Sprague-Dawley rats (220 - 240 g, Experimental Animal Center of Shanghai Medical University, Grade Ⅱ, Certificate No 02-

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Apr; 20 (4)
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22-2) were anesthetized with sodium pentobarbital (40 mg·kg^{-1}, ip), perfused intracardiacally with normal saline, followed by 4 % fresh paraformaldehyde in phosphate-buffer saline 0.1 mol·L^{-1} (PBS, pH = 7.4). Serial coronal slices (30 μm) of brain were made on a freezing microtome. The sections were stored in the modified cryoprotectant solution at −20 ℃.

Sections were used for immunohistochemistry. The sections were washed with PBS, pretreated with 0.3 % hydrogen peroxide for 10 min, incubated with 10 % normal goat serum/0.3 % Triton X-100 for 30 min, rabbit anti-VEGF (1∶1000 from Santa Cruz Biotechnology, Inc) at 4 ℃ overnight, biotinylated goat anti-rabbit IgG (ABC kit from Vector Lab) at 1∶200 dilution for 30 min, avidin-biotin-peroxidase (from ABC kit) at 1∶100 dilution for 45 min, and stained with 3, 3′-diamino-benzidine/hydrogen peroxide (DAB from Sigma). Immunocytochemical control sections received the same treatment except exposure to the primary antibody and showed no specific staining.

Cannula placement Sprague-Dawley rats (220 − 250 g, Experimental Animal Center of Shanghai Medical University, Grade Ⅱ, Certificate № 02-22-2) were peritoneally anesthetized with 10 % chloral hydrate (360 mg·kg^{-1}), and stereotaxically implanted with guide cannula (OD 0.8 mm; 2-cm long) into the lateral cerebral ventricle at Bregma −0.8 mm; R 1.4 mm; H 4.0 mm. The guide cannula was secured to skull with dental cement. After a 3-day recovery period, rats were subjected to cerebral ischemic operation.

Focal cerebral ischemia model Three days after cannula implantation, rats were weighed again, and anesthetized with 10 % chloral hydrate (360 mg·kg^{-1}). Then the focal ischemia model was induced which was largely based on method described by Longa[10]. In brief, Sprague-Dawley rats (220 − 250 g) were peritoneally anesthetized with 10 % chloral hydrate (360 mg·kg^{-1}), and a tail artery was cannulated for monitoring blood pH, p_{CO_2} and p_{O_2}, which were within the physiological range. The rectal temperature was maintained at (37 ± 0.5) ℃ by heating pad and heating lamp. Under a dissecting microscope, the superior thyroid and pterygopalatine arteries were isolated and coagulated, and the occipital artery was ligated. A 4-0 monofilament nylon suture was introduced into the lumen of the internal carotid artery for a length about 22 mm for 1 h.

Experimental protocol Rats were subjected to ischemic surgery and randomly divided into 2 groups: the control group was treated with vehicle (rabbit IgG 0.1 g·L^{-1} daily); the antibody group was treated with VEGF antibody (0.1 g·L^{-1} daily). VEGF antibody or rabbit IgG was injected icv between 8:30 − 9:30 am every day following the ischemia for 7 d.

Infarct volume assessment Seven days after cerebral infarction, rats were decapitated. Brains were immersed in cold saline, and were sectioned into six standard coronal slices (2-mm thick). Slices were placed in the vital dye 2 % TTC at 37 ℃ in the dark for 30 min, and fixed by 10 % formalin at 24 ℃ overnight.

Statistics Infarct volume of each brain slice was analyzed by computer image analyzing system. Infarct volumes in vehicle versus VEGF antibody-treated rats were compared by two-tailed t-test for each experiment. Data were expressed as $\bar{x} \pm s$.

RESULTS

Distribution of VEGF in adult rat brain VEGF protein was widely expressed in the adult rat brain. Immunohistochemistry showed that positive staining was restricted to neuronal cytoplastic perinuclear regions and principal dendrites. The densities of VEGF-like positive staining in various brain areas were detected by microscopic image analyzer (Fig 1). The intensity of reaction varied with the location of the neurons. The highest densities of VEGF-like positive staining cells were found in the granule cell layer of dentate gyrus. The medium densities were seen in the pyramidal layer of neocortex, cingulum, piriform, entorhinal, septal, the diagonal band of broca, the pyramidal layer of hippocampus, nucleus of ventromedial thalamus, ventroposterior thalamus, paraventricular hypothalamus, arcuate hypothalamus, and ventromedial hypothalamus. The lowest densities were noted in the amygdaloid nucleus, nucleus accumbens, subiculum, nucleus of preoptic hypothalamus, mammillary body, and geniculate body. There was no positive staining

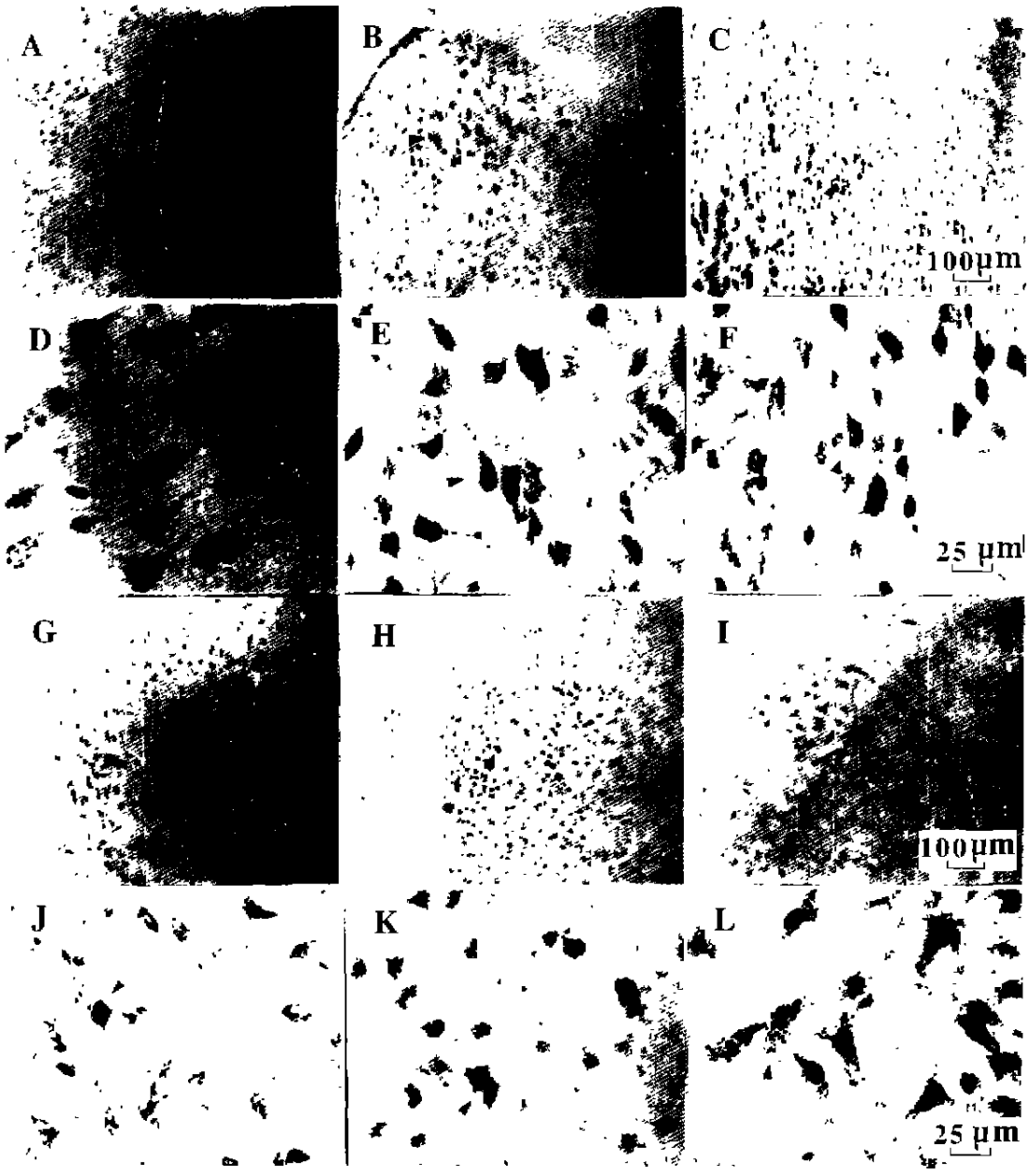

Fig 1. Neuronal distribution of VEGF in the adult rat brain ($n = 1$).
Photographs show low-(A – C, G – I) and high-(D – F, J – L) power magnifications of representative fields on coronal sections. A) Cingulate cortex, B) Dorsal septal nucleus, C) Medial septal nucleus, G) Geniculate body, H) Basomedial amygdaloid, I) Substantia nigra.

in the neocortex of molecular layer, external layer, inner granular layer, ganglion layer, and multiform layer. (Tab 1)

Furthermore, the expression of VEGF was time-dependently increased in the neurons in the MCA territory following the cerebral ischemia. Throughout the experiment, neurons in the areas remote from the ischemic territory showed no apparent immunoreactivity

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Apr; 20 (4)
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Tab 1. The density of neuronal distribution of VEGF in adult rat brain ($n = 3$).

Brain regions	Density
Ⅰ Telencephalon	
Cerebral cortex	
Molecular layer	−
External layer	−
Pyramidal layer	+ +
Inner granular layer	−
Ganglion layer	−
Multiform layer	−
Cingulum	+ +
Piriform	+ +
Entorhinal	+ +
Amygdala nucleus	+
Septal later, medial area	+ +
Striatum	
Caudate putamen	+ / −
Nucleus accumbens	+
The diagonal band of broca	+ +
Hippocampal complex	
Dentate gyrus	+ + +
Hippocampus	
CA1	+
CA2	+ +
CA3	+ +
CA4	+ +
Subiculum	+
Ⅱ. Diencephalon	
Thalamus	
Ventromedial thalamic nucleus	+ +
Ventroposterior thalamic nucleus	+ +
Hypothalamus	
Paraventricular	+ +
Preoptic	+
Ventromedial nuclei	+ +
Mammillary body	+
Arcuate	+ +
Ⅲ. Brainstem	
Substantia nigra	+ +
Geniculate body	+

−) 0; +) $< 29 \times 10^3$ A/mm^2; + +) $(30 - 59) \times 10^3$ A/mm^2; + + +) $> 60 \times 10^3$ A/mm^2

change of VEGF compared with controls (unpublished data).

Effects of VEGF antibody on the neuronal damage induced by focal cerebral ischemia

VEGF antibody ($n = 11$) or vehicle ($n = 7$) was injected icv daily following the ischemia for 7 d. Infarct volume in the antibody treatment was increased versus vehicle-treated rats [$(21.6 \pm 2.7$ *vs* $16 \pm 6)$ mm^3, $P < 0.05$] respectively (Fig 2).

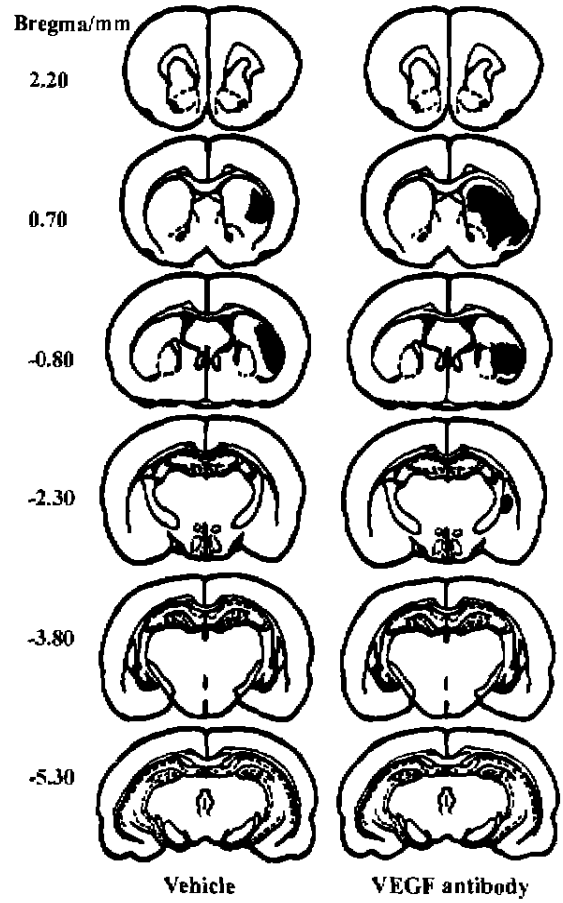

Fig 2. Coronal slices in a rat brain reperfusion 7 d after 1-h MCA occlusion. Black areas express infarct stained with TTC.

DISCUSSION

Our results demonstrate that VEGF is widely expressed in many regions in the adult rat brain, which seems not directly relating to the distribution of the cerebral blood vessels according to the morphological characteristic of positive staining cells. Analysis by morphological appearance showed that it seemed important that neurons expressed VEGF in the central nervous system, although VEGF has been shown to play a role in mediating the increase of both capillary permeability and angiogenesis. Neuronal distribution of VEGF in the mature brain may reflect that VEGF played possible roles in neurophysiological process in the central nervous system, since the vascularization was completed, the rate of endothelial cell proliferation was very low, and the blood-brain-barrier was intact in the adult rat brain[11].

In our previous study, we observed that VEGF was time-dependently expressed in the neuronal cells of rat brain after a transient cerebral ischemia[9]. Especially, in the hippocampal CA2 and CA3 areas, the cells which are more resistant to the ischemia and are destined to survive were observed to highly express VEGF. However, in the hippocampal CA1 area, the cells, which are vulnerable to cerebral ischemia or traumatic brain injury and are destined to die were not observed to express VEGF[12]. Recent studies of permanent and transient MCAO in rats also showed increase of VEGF protein in neurons[13,14]. In the present paper, we obviously observed that VEGF antibody could enhance an infarct volume induced by ischemia, which was consistent with the response of ischemic rats to chronic infusion of VEGF into the ischemic core as recently reported by Hayashi *et al*[15]. Putting together, it suggests that increased expression of VEGF in the neurons may be a beneficial factor for cell survive following the ischemia.

Besides VEGF could be expressed in the neurons around ischemic penumbra areas, it was also expressed in the endothelium in the ischemic core. However, whether VEGF can protect cerebral tissues from ischemia through formation of new capillary collateral channels still needs to be further elucidated.

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ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Apr; 20(4)
E-mail aps@server.shcnc.ac.cn Phn Fax 86-21-6474-2629

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侧脑室注射血管内皮生长因子抗体加重短暂性脑缺血后脑损伤[1]

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关键词 短暂性脑缺血；再灌注损伤；血管内皮；内皮生长因子；免疫组织化学

目的：研究血管内皮生长因子(VEGF)在脑缺血再灌注引起的神经损伤中的作用. **方法**：用免疫组织化学方法观察 VEGF 在 SD 成年大鼠脑内的表达；用大鼠大脑中动脉阻死再灌注模型，结合 TTC 染色和计算机图象分析测量脑梗死体积，观察 VEGF 抗体对缺血性脑梗死体积的影响. **结果**：成年大鼠脑内的神经元上有大量 VEGF 表达，此表达具有脑区分布的特异性；侧脑室注射 VEGF 抗体，脑梗塞体积为(21.6±2.7) mm^3，对照组为(16±6) mm^3($P<0.05$). **结论**：侧脑室注射 VEGF 抗体加重脑缺血再灌注后脑损伤，提示神经元表达的 VEGF 可能对缺血的脑组织有保护作用.

(责任编辑 李 颖)

关于申报评选 1999 年第三届 中国药理学会 Servier 青年药理学工作者奖的通知

经中国药理学会与法国 Servier 研究院商定，现将 1999 年第三届中国药理学会 Servier 青年药理工作者奖申报评选工作的通知：

1 获奖候选人条件：

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1.2 年龄在 37 岁(1962 年 10 月 31 日以后出生)以下；

1.3 在国内从事药理学研究并取得优秀成绩，不包括在国外做过的工作；

1.4 从获奖之日起至少在国内工作一年以上.

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2.2 初审各地评选负责人(或学会)组织专家对本地区申报材料进行评审，评选出 3 名候选人(西南、重庆可多报 1－2 名)，将其材料及本地区专家组评审意见及排名顺序，于 1999 年 8 月底前报送中国药理学会办公室(100050 北京先农坛街 1 号中国药理学会孙静霞收)

2.3 终审：由中国药理学会组织专家评审，选出 12－16 名候选人送 Servier 法方专家评审. 9 月由中法两国评委共同评出 8 名获奖者.

3 颁奖：

1999 年 10 月在西安"中国药理学会世纪之交学术会议"期间举行颁奖活动，将发给每位获奖者荣誉证书和壹万元奖金.

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