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Expression of angiopoietin-2 and vascular endothelial growth factor in mice cerebral cortex after permanent focal cerebral ischemia[1]

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KEY WORDS endothelial growth factors; angiopoietin-1; angiopoietin-2; Tie-1; Tie-2; inbred C57BL mice; brain ischemia; reverse transcriptase polymerase chain reaction; messenger RNA; immunohistochemistry

ABSTRACT

AIM: To study the expressions of vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), Tie-1, and Tie-2 in C57BL/6 mouse brain after permanent focal cerebral ischemia. **METHODS**: The mRNA levels of VEGF, Ang-1, Ang-2, Tie-1, and Tie-2 were measured by semiquantitative reverse transcription polymerase chain reaction (RT-PCR). The protein expressions of VEGF and Ang-2 were determined by immunohistochemistry. **RESULTS**: Low mRNA levels of VEGF, Ang-1, Ang-2, Tie-1, and Tie-2 were constitutively expressed in the normal cortex of mouse. After middle cerebral artery occlusion (MCAO), the expressions of VEGF, Ang-2, and Tie-2 mRNA were dramatically increased in the infarcted cortex and the elevation was remained through 7 d of ischemia. However, the levels of Ang-1 and Tie-1 mRNA were unchanged in the infarcted cortex. Immunoreactivities of Ang-2 or VEGF were hardly observed in the normal cortex. Ang-2 protein was evidently detected in the infarct core 8 h after MCAO and in the perifocal area 1 d after MCAO. Expression of VEGF protein was elevated in the infarct core 2 h after MCAO and in the perifocal area 1 d after MCAO. Immunoreaction was restricted to endothelial cells and glial-like cells within the infarct core and perifocal area. **CONCLUSION**: The expressions of Ang-2 and VEGF are induced after focal cerebral ischemia, which may contribute to the angiogenic response in the cortex of ischemic brain.

INTRODUCTION

Angiogenesis, the formation of new blood vessels by spouting of endothelial cells from pre-existing vessels, occurs not only during physiological processes but also in pathological settings[1,2]. For example, in stroke patients at autopsy, the number of microvessels in the infarcted brain tissue was increased compared with the contralateral normal hemisphere, and higher blood vessel counts correlated with longer survival[3]. In addition, induction of endothelial cell proliferation and new vessel growth has also been demonstrated in ischemic cortex after focal cerebral ischemia in mice[4].

The molecular mechanisms underlying angiogenesis have been extensively characterized. It has been found that vascular endothelial growth factor (VEGF) and angiopoietin (Ang) could modulate angiogenesis in the experimental conditions. VEGF is a potent angiogenic factor and it binds to two endothelial tyrosine kinase receptors, VEGF receptor-1 (VEGFR-1, Flt-1) and VEGFR-2 (Flk-2/KDR). Ang-1 and its naturally occuring antagonist Ang-2 specifically bind to endothelial tyrosine kinase receptor Tie-2[5,6]. They are essential for normal vascular development. VEGF and Ang-2 are up-regulated strongly by hypoxia. In addition, *in vivo* experiments have revealed that VEGF and Ang-2 expression is clearly induced in hypoxic areas in various models of ischemia[4,7-10]. Furthermore, the VEGF/VEGFR system induced by hypoxia leads to the growth of new vessels after cerebral ischemia[4]. It has been suggested that VEGF and Ang-2 might be two crucial effectors of hypoxia-induced neovascularization.

In the present study, we observed the expression of these factors in the infarct cortex using a middle cerebral artery occlusion (MCAO)-induced permanent focal ischemic mouse model.

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MATERIALS AND METHODS

Drugs and reagents Agarose, ethidium bromide, and diaminobenzidine were purchased from Sigma Chemicals Co (USA). TRI-REAGENT-LS extraction kit was purchased from Molecular Research Center Inc (USA). RNasin, dNTP, oligo $(dT)_{18}$ primer, and *Taq* DNA polymerase were obtained from Sangon Biotechnology Co (Canada). M-MuLV reverse transcriptase was from Fermentas Inc (Lithuania). Goat polyclonal antibodies to Ang-2 or VEGF were purchased from Santa Cruz Co (USA). Biotinylated secondary antibody and streptavidin-peroxidase complex were obtained from Wuhan Boster Biological Technology Co (China).

Animals C57BL/6 male mice (n = 50, Grade Ⅱ, Certificate № 003, weighing 25 g ± 2 g) were obtained from Shanghai Experimental Animal Center, Chinese Academy of Sciences. They were kept in a temperature (25 ℃ ± 2 ℃) and humidity (50 % ~ 70 %) controlled room, with a daily light cycle before and after surgery. All mice had free access to food and water.

Permanent focal cerebral ischemic model Permanent focal cerebral ischemia was achieved by electrocoagulation of the left middle cerebral artery (MCA) as previously described[11]. Briefly, mice were anesthetized with chloral hydrate (500 mg/kg, ip). A skin incision was made between the orbit and ear. The superior pole of the parotid gland was reflected downward, as was the temporalis after partial resection of its cranial insertion. The distal course of the MCA was then visible through the translucent skull. A small burr-hole craniectomy was performed with a dental drill. The MCA was coagulated by bipolar diathermy. The muscle and soft tissue were replaced and the incision was sutured. Mice were placed in a warm environment until they recovered from anesthesia. This procedure results in a reproducible ischemic lesion restricted to the temporoparietal cortex (ipsilateral to surgery) of operated animals. For sham operations, the MCA was exposed in the same way but not occluded.

Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) At different time intervals (8 h, 1 d, 2 d, 4 d, and 7 d) after MCAO or sham operation (1 d), mice were decapitated and the whole brains were removed. Both ischemic (the cortex ipsilateral to surgery) and contralateral cortices from the same mouse were dissected. Nonoperated normal mice were used as control. Total RNA was prepared from cortical samples with TRI-REAGENT-LS extraction kit according to the manufacturer's guidelines. For cDNA synthesis, 20 μL reverse transcription mixture containing total RNA 1 μg, $MgCl_2$ 10 mmol/L, dNTP 2 mmol/L, oligo $(dT)_{18}$ primer 1 μg, RNasin 20 U, M-MuLV reverse transcriptase 20 U was incubated at 37 ℃ for 90 min and then the reverse transcriptase was inactivated by heating the reaction mixture to 95 ℃ for 10 min.

PCR was performed to assess the expression of Ang-1, Ang-2, Tie-1, Tie-2, and VEGF mRNA using β-actin as an internal control. Oligonucleotide primers (Tab 1) specific for mouse Ang-1, Ang-2, Tie-1, Tie-2, VEGF, and β-actin were synthesized according to published sequences. PCR conditions were as follows: 1 μL of cDNA mixture was subjected to amplification in 50 μL of final volume with $MgCl_2$ 2.5 mmol/L, dNTPs 200

Tab 1. *Oligonucleotide primers used in PCR.*

Gene		Primers	Product size bp
VEGF	Sense	5′ GCGGGCTGCCTCGCAGTC 3′	716 ($VEGF_{188}$), 644 ($VEGF_{164}$),
	Antisense	5′ TCACCGCCTTGGCTTGTCAC 3′	512 ($VEGF_{120}$)
Ang-1	Sense	5′ GACAGAGCAGTACAACACCA 3′	362
	Antisence	5′ GGAGAAGTTGCTTCTCTAGC 3′	
Ang-2	Sense	5′ CGCATTCGCTGTATGATCAC 3′	447
	Antisence	5′ GCACTTCCTGATGTGGAAAG 3′	
Tie-1	Sense	5′ CCTATATCCAAGTACATCGTGG 3′	417
	Antisence	5′ GAACTGCAGGATGGTCTCTTCA 3′	
Tie-2	Sense	5′ GATCTCCAACATCACTGACTCC 3′	458
	Antisence	5′ TTCCTGAGTTAAACTGCACAGC 3′	
β-actin	Sense	5′ TCAGAAGGACTCCTATGTGG 3′	500
	Antisence	5′ TCTCTTTGATGTCACGCACG 3′	

μmol/L, 3 U of *Taq* DNA polymerase, and 50 pmol of each primer in the reaction buffer. For all of the reactions, preliminary experiments were performed to determine the number of PCR cycles at which saturation occurred, and the experiments mentioned were carried out with a number of cycles that precedes saturation. PCR cycles were as follows. Ang-1 and Ang-2 primers: 95 ℃, 5 min; 94 ℃, 45 s; 55 ℃, 45 s; 72 ℃, 90 s (32 cycles); 72 ℃, 10 min. VEGF primers: 95 ℃, 5 min; 94 ℃, 45 s; 65 ℃, 45 s; 72 ℃, 90 s (30 cycles); 72 ℃, 10 min. Tie-1 and Tie-2 primers: 95 ℃, 5 min; 94 ℃, 45 s; 59 ℃, 45 s; 72 ℃, 90 s (32 cycles); 72 ℃, 10 min. β-Actin primers: 95 ℃, 5 min; 94 ℃, 45 s; 55 ℃, 45 s; 72 ℃, 90 s (25 cycles); 72 ℃, 10 min. PCR was performed on a thermocycler PTC-150 (MJ, USA). The absence of contamination was routinely checked by RT-PCR on negative control samples in which either the RNA samples were replaced with sterile water or the M-MuLV reverse transcriptase was omitted. No specific cDNA was obtained using these conditions. PCR products 10 μL were separated by 1.8 % agarose gel electrophoresis and visualized using ethidium bromide staining. The density of each band was measured by a densitometer. This semiquantitative measure was expressed as ratios compared with β-actin.

Light microscopic immunohistochemistry After mice were anesthetized with chloral hydrate 500 mg/kg ip, they were perfused transcardially with 200 mL of PBS (Na_2HPO_4 10 mmol/L, 0.84 % NaCl, pH 7.4), followed by 200 mL of 4 % formaldehyde in PBS. The brain was removed and post-fixed by 4 h immersion in 4 % formaldehyde in PBS at 4 ℃. The tissue was cryoprotected by an overnight immersion in sucrose (30 % sucrose, Na_2HPO_4 10 mmol/L, pH 7.4, 4 ℃), frozen on dry ice, and then cut at −20 ℃ in a cryostat. Sections were collected on warm, poly-*D*-lysine-coated slides. For immunohistochemistry, the tissue sections were incubated with goat polyclonal antibodies to Ang-2 or VEGF (1∶100 dilution) at 4 ℃ overnight in a moist chamber. Biotinylated secondary antibody was then added and incubated at 25 ℃ for 20 min. The streptavidin-peroxidase complex was subsequently added for another 20 min at 25 ℃. The peroxidase activity was visualized with 0.003 % diaminobenzidine and 0.001 % H_2O_2 in PBS.

Statistics Data were expressed as $x \pm s$, and compared with student t-test. Each group was performed in triplicate.

RESULTS

Expression of VEGF, Ang-2, and Tie-2 mRNA in brain cortex induced by focal ischemia Semiquantitative RT-PCR with β-actin as an internal standard was used to characterize the temporal profiles of Ang-1, Ang-2, Tie-1, Tie-2, and VEGF mRNA expression in the infarct cortex (Fig 1). Low levels of Ang-1, Ang-2, Tie-1, Tie-2, and VEGF mRNA were constitutively expressed in the normal brain cortex. No significant change was found in the expression of Ang-1 and Tie-1 mRNA in the ipsilateral cortex after occlusion MCAO caused a time-related increase of VEGF, Ang-2, and Tie-2 expression in the ischemic cortex. The levels of VEGF, Ang-2, and Tie-2 mRNA were up-regulated 8 h after occlusion and increased to 4.7, 12.8, and 6.8 folds compared to nonoperated normal mice, respectively, at 1 d. And this elevation was remained through d 7 after the onset of cerebral ischemia.

Ang-2 and VEGF immunoreactive cells in the core and perifocal regions The expression of Ang-2 and VEGF protein was detected in the ischemic cortex using immunohistochemistry. Ang-2 and VEGF were hardly detected in the cerebral cortex of nonoperated normal mice (Fig 2A, Fig 3A). There was a slight Ang-2 immunostaining in the infarct core 2 h after MCAO (Fig 2B). Ang-2-positive cells with numerous and intense immunoreaction occurred in the core 8 h later (Fig 2C, 2D, 2F). Cells with strong Ang-2 immunostaining appeared in the perifocal regions 1 d after MCAO (Fig 2E, 2G). The strongest immunostaining of VEGF occurred in the infarct core only 2 h after MCAO (Fig 3B) and after then VEGF immunoreactivity became weak (Fig 3C, 3D, 3F). One day after MCAO, VEGF immunostaining appeared in the regions around the infarct (Fig 3E, 3G). The cells with Ang-2 or VEGF immunoreactivity were endothelial cells and glial-like cells (Fig 2H, 3H).

DISCUSSION

Hypoxia is an important driving force for angiogenesis during cerebral ischemia. Although intensively studied, the mechanisms involved in the process of hypoxia-induced angiogenesis are only partially known. VEGF is distinctive in that its mitogenic effect is highly specific for endothelial cells and its expression is up-regulated by hypoxia. In the present study, VEGF mRNA expression was elevated in the ischemic cerebral

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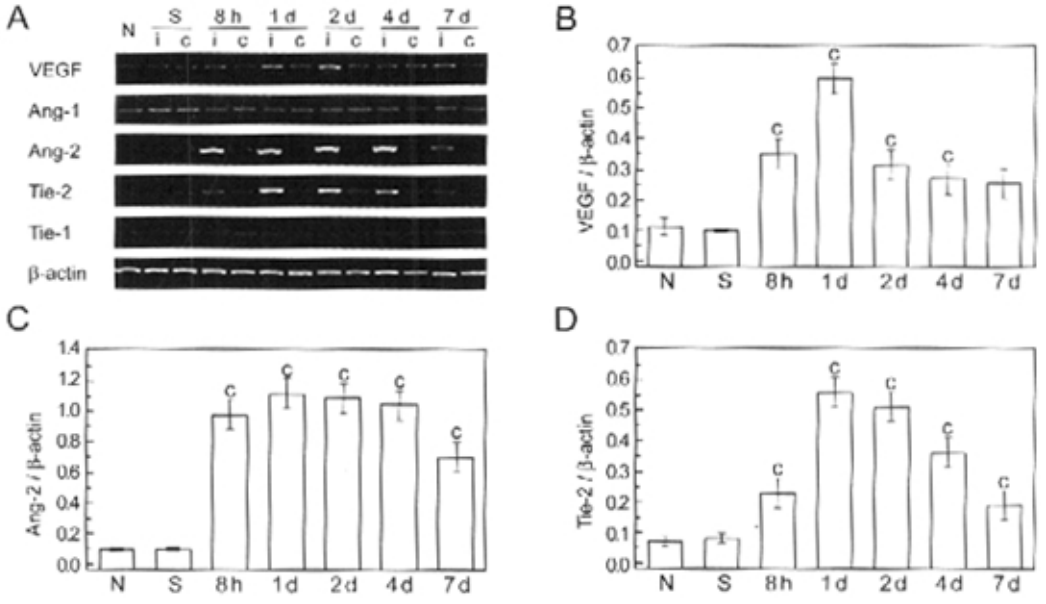

Fig 1. RT-PCR analysis of VEGF, Ang-2, and Tie-2 mRNA in C57BL/6 mice cortex. cDNA was synthesized from 1 μg of total RNA from contralateral (c) and ipsilateral (i) brain hemispheres at different time after MCAO. PCR products were separated by 1.8 % agarose gel electrophoresis and visualized using ethidium bromide staining. A) Representative of results obtained from three individual experiments. B), C), D) The ratio of VEGF/β-actin, Ang-2/β-actin, and Tie-2/β-actin were used to express the mRNA levels of VEGF, Ang-2, and Tie-2 in mouse cortex after MCAO, respectively. N, normal; S, sham operation. $n = 3$ mice for each group. $\bar{x} \pm s$. $^cP < 0.01$ *vs* N.

cortex after permanent MCAO. And the increase of VEGF protein was also detected in the infarct core and in the penumbra areas. These findings are consistent with other reports[4,7].

The discovery of Ang-1 and Ang-2 has provided novel and important insights into the molecular mechanisms of angiogenesis[5,6]. Ang-1, the major physiological agonist of Tie-2, is essential for formation and maturation of the vessels[5]. Ang-1 could also be a key molecule for the maintenance of blood vessel stability in the adult[12]. Ang-2, the natural Ang-1 antagonist, might promote a process of vessel wall disassembly by blocking Ang-1 induced Tie-2 phosphorylation and could be an important pro-angiogenic factor[6]. In the present study, the level of Ang-2 mRNA but not Ang-1 mRNA was elevated in the ischemic cortex. Expression of Tie-2 mRNA but not Tie-1 mRNA was also increased. An increase of Ang-2 protein was also observed in the infarct cortex. And importantly, expression of Ang-2 protein in the perifocal regions was elevated simultaneously with that of VEGF. It has been reported that up-regulation of both Ang-2 and VEGF coincided with endothelial cell proliferation[10] and that the number of newly formed vessels strongly increased at the border of the infarct[1]. Thus, Ang-2/VEGF pair might be the key effector of angiogenesis in the ischemic brain.

Involvement of Ang/Tie-2 system in the process of vessel regression has also been reported. Ang-2 as an Ang-1 antagonist could be a key factor for vascular cell apoptosis by blocking the effect of Ang-1[13]. In the present study we found that VEGF protein increased quickly in the infarct core 2 h after MCAO and then reduced gradually. Ang-2 protein was expressed intensely in the infarct core 8 h after MCAO and the elevation was remained through d 2. Therefore, it is possible that at some time ie 2 d after MCAO, augmentation of Ang-2 may induce the regression of vascular in the ischemic core because of the reduction of VEGF protein expression.

In conclusion, Ang-2 and VEGF mRNA expressions were increased in the ischemic cerebral cortex after MCAO, the time course of induced Ang-2 protein expression in the infarct core was different from that of VEGF protein, and Ang-2 protein expression coincided

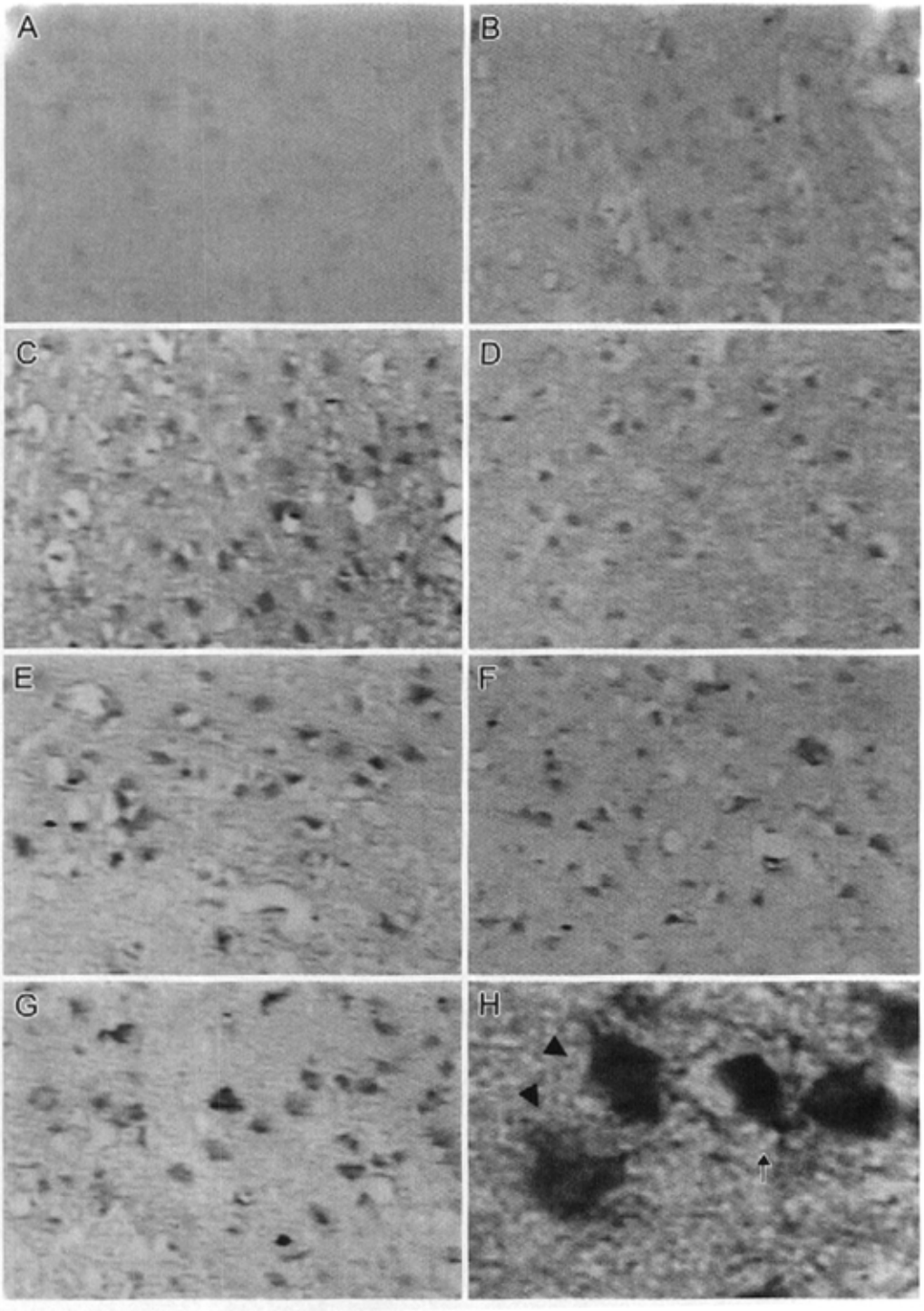

Fig 2. Immunoreactivity to Ang-2 protein in C57BL/6 mice cortex at different time points after MCAO. A) Normal, × 100. B), C), D), F) Infarct core of 2 h, 8 h, 1 d, 2 d after MCAO, × 100. E), G) Perifocal area of 1 d and 2 d after MCAO, × 100. H) The immunostaining was restricted to endothelial cells (arrow) and glial-like cells (bold arrow head), × 400.

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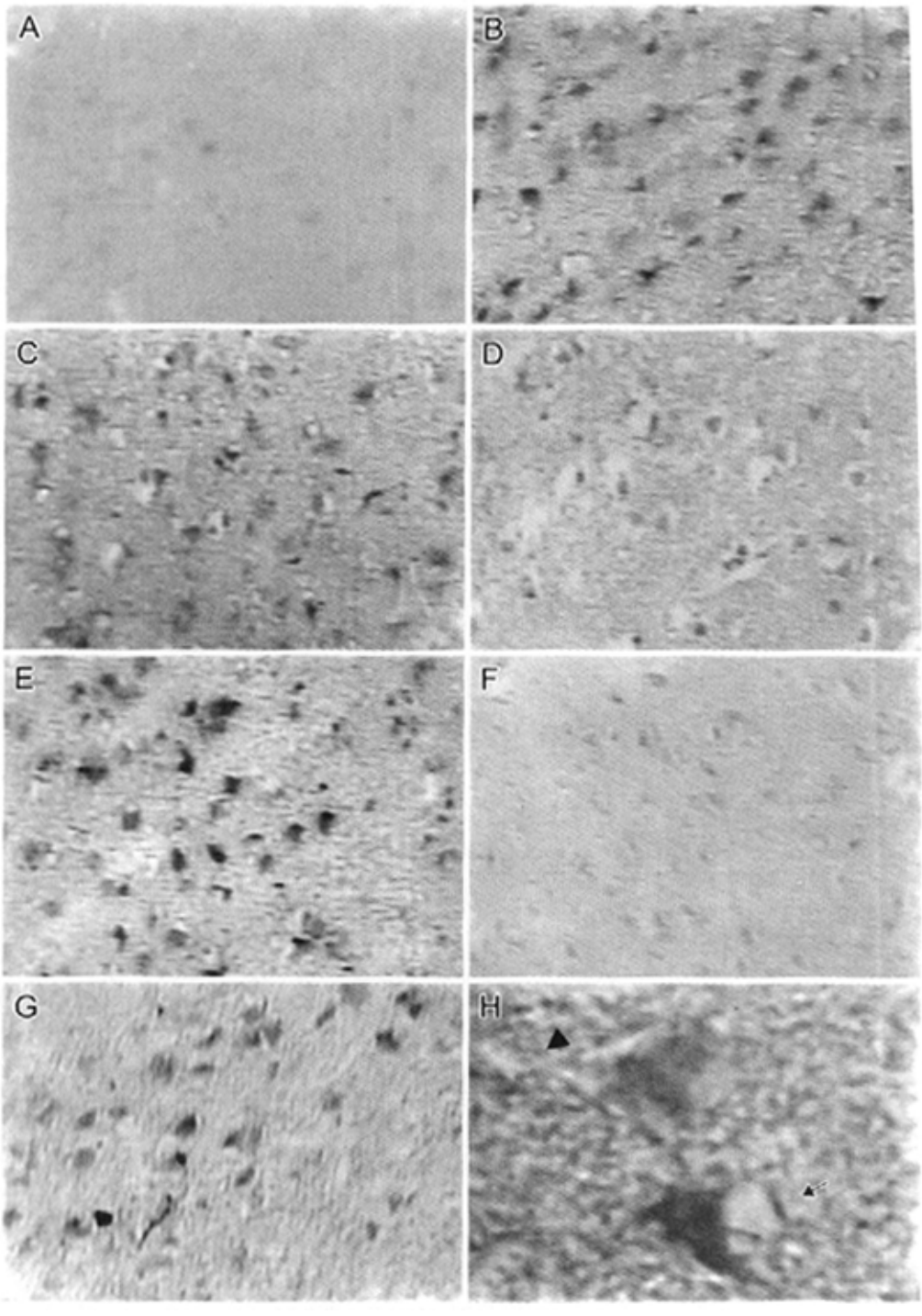

Fig 3. Immunoreactivity to VEGF in C57BL/6 mice cortex at different time points after MCAO. A) Normal, × 100. B), C), D), F) Infarct core of 2 h, 8 h, 1 d, 2 d after MCAO, × 100. E), G) Perifocal area of 1 d and 2 d after MCAO, × 100. H) The immunostaining was restricted to endothelial cells (arrow) and glial-like cells (bold arrow head), × 400.

with VEGF protein in the perifocal areas. These data suggested that Ang-2 and VEGF might be involved in vessel regression of ischemic core, proliferation of endothelial cells, and growth of new vessels in perifocal regions.

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永久性大脑局灶缺血后 angiopoietin-2 和血管内皮生长因子在小鼠大脑皮层的表达[1]

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关键词 内皮生长因子；angiopoietin-1；angiopoietin-2；Tie-1；Tie-2；近交 C57BL 小鼠；脑缺血；逆转录聚合酶链反应；信使 RNA；免疫组织化学

目的：研究永久性大脑局灶缺血后血管内皮生长因子(VEGF)、angiopoietin-1 (Ang-1)、Ang-2、Tie-1 和 Tie-2 在 C57BL/6 小鼠大脑皮层的表达. **方法**：采用半定量逆转录聚合酶链反应(RT-PCR)研究 VEGF, Ang-1、Ang-2、Tie-1 和 Tie-2 mRNA 表达的变化. 采用免疫组织化学法研究 VEGF 和 Ang-2 蛋白的表达. **结果**：在正常小鼠大脑皮层 VEGF、Ang-1、Ang-2、Tie-1 和 Tie-2 mRNA 表达水平很低. 当大脑中动脉阻断(MCAO)后，在梗塞皮层 VEGF、Ang-2 和 Tie-2 mRNA 表达显著增加，并且可维持到第 7 天. 但 Ang-1 和 Tie-1 mRNA 表达在梗塞皮层无明显变化. 在正常小鼠大脑皮层，几乎观察不到 VEGF 和 Ang-2 蛋白的表达. MCAO 后 8 小时 Ang-2 蛋白表达在皮层梗塞区明显增加，1 天后可在梗塞灶周边区看到 Ang-2 蛋白增加；而 VEGF 蛋白则在 MCAO 后 2 小时在皮层梗塞区即显著升高，1 天后在梗塞灶周边区可观察到 VEGF 蛋白增加. 不论在皮层梗塞区还是在梗塞灶周边区，Ang-2 和 VEGF 免疫阳性都限于内皮细胞和胶质样细胞. **结论**：大脑局灶缺血后 Ang-2 和 VEGF 表达增加，可能有利于缺血大脑皮层的血管新生.

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