Induction of Angiogenesis Related Genes in the Contralateral Cortex with a Rat Three-Vessel Occlusion Model

Wai-mui Cheung, Shu-fen Chen, Gau-ming Nian and Teng-nan Lin

Division of Neuroscience
Institute of Biomedical Sciences
Academia Sinica
Taipei 115, Taiwan, ROC

Abstract

The bFGF/FGFR, VEGF/VEGFR and Angiopoietin/Tie receptor system are crucial for angiogenesis and vascular remodeling. With a rat focal cerebral ischemia model, we previously reported dramatic changes in the vascular density and angiogenesis related genes in the ipsilateral cortex after 60 minutes severe ischemia. While only a small increase in the capillary density was noted in the contralateral cortex with very mild ischemia. In the present study we further reported that only Tie-1 and VEGFR-2 mRNA were significantly changed in the contralateral cortex with a p value of 0.0001 and 0.0168, respectively, and the degree of changes were very small. Interestingly, in contrast to a huge increase in the ipsilateral cortex, Tie-1 mRNA was slowly decreased after the onset of ischemia and stayed below the basal level throughout the remaining periods studied. The mechanism and significance for this decrease is not presently clear. In contrast to the ipsilateral cortex, the Angpo-1/Angpo-2 mRNA ratio was also slightly dropped below the basal level in the contralateral side in most of the ischemia-reperfusion periods studied, which is in line with the notion that small decrease in Angpo-1/Angpo-2 mRNA ratio implied small vascular remodeling activity. It is very likely that increase in this Angpo-1/Angpo-2 ratio is crucial for remodeling into large vessels and increase in Tie-1 may be crucial for capillary density increasing. Nevertheless, the detailed mechanisms and significance of differential expression of these genes and relationship to vascular remodeling remain to be characterized.

Key Words: angiopoietin, tie, bFGF, VEGF, stroke

Introduction

The development of vascular supply is a fundamental requirement for organ development and differentiation during embryogenesis as well as for wound healing, and reproductive functions in the adult (4). Angiogenesis has also been implicated in the pathogenesis of a variety of disorders: proliferative retinopathies seen in age-related macula degeneration, tumors, rheumatoid arthritis and psoriasis. Ischemic brain injury is a consequence of severe reduction of blood supply to the affected region. The resultant low tissue oxygen tension often leads to compensatory neovascularization to meet the metabolic demand (12). Examples include the development of collateral blood vessels to the ischemic region and angiogenesis in the healing of hypoxic wounds. Angiogenesis occurs in the ischemic brain, particularly in the ischemic penumbra. The extent of angiogenesis has been correlated to survival in stroke patients (6). However, the presumed hypoxia-induced angiogenic factors that mediate this compensatory response have not been fully identified. The putative factors include VEGF, bFGF and angiopoietin (5, 9, 10). All were up-regulated after focal cerebral ischemia with a time course that parallels angiogenesis.

Recently, Zhang et al. (15) reported that Tie-1 mRNA was up regulated on cerebral micro-vessels

Corresponding author: Dr. Teng-nan Lin, Division of Neuroscience, Institute of Biomedical Sciences, Academia Sinica, Taipei 115, Taiwan, ROC. Fax: 886-2-2785-8594, E-mail: bmltn@ibms.sinica.edu.tw
Received April 27, 2000; Revised June 1, 2000; Accepted June 3, 2000.

after embolic MCA occlusion. We previously reported that not only Tie-1 mRNA, Tie-2 mRNA and its ligands, Angpo-1 and Angpo-2 mRNAs, were also up regulated in the ipsilateral cortex after 60 minutes ischemia (10). The temporal induction profiles of VEGF/VEGFR, bFGF/FGFR and angiopoietin/Tie genes were different, which suggest the involvement of complex regulatory mechanisms for ischemia-induced angiogenesis that remain to be characterized. The expression patterns of these genes could be related to progressive neovascularization after ischemia in this stroke model. Indeed, a marked increase in the vascular density was noted in the ipsilateral side. Interestingly, a substantial mild increase in the capillary density was also noted in the contralateral cerebral cortex, which suffered very mild ischemia. The purpose of the present study is to study the expression of angiogenesis related genes in the contralateral cerebral cortex, which was not report in the previous study. A comparison on those induction profiles in the ipsilateral side, mild vs. severe, may lead to better understanding of their role in angiogenesis during ischemia-reperfusion.

Materials and Methods

Stroke Model

The focal cerebral ischemia-reperfusion model in the rat has been described previously (7). In brief, male Long-Evans rats weighing 250 to 300 gs were anesthetized with chloral hydrate (360 mg/kg body weight, i.p.). The trunk of the right middle cerebral artery (MCA) above the rhinal fissure was identified under a stereomicroscope and ligated with a 10-0 suture. Interruption of blood flow distal to ligation was confirmed under the microscope. Both common carotid arteries were then occluded using nontraumatic aneurysm clips. After 60-min of ischemia, the aneurysm clips and the suture were removed and restoration of blood flow in all three arteries was verified. In this stroke model, only the right MCA cortex sustained severe ischemia with regional blood flow reduction by 88% to 92% (n=4). Only very mild ischemia was noted (reduction of blood flow by 10 to 20 %) out side the right MCA cortex (1). While under anesthesia, the rectal temperature was monitored and maintained at 37.0±0.5°C using a homeothermic blanket (Harvard, USA). In this model, ischemia for 60-min consistently resulted in a large infarct confined to the right MCA cortex (11). No morphological (11) or biochemical evidence of ischemic brain injury was noted in the left MCA cortex (1). Following the ischemic insult, rats were kept in an air-ventilated incubator at 24.0±0.5°C for up to 2 weeks and were provided with water and lab chow ad libitum until the end of experiments. At the end of each experiment (30 [-0.5] and 60 [0] minutes after onset of ischemia or 0.5, 1, 1.5, 4, 12, 24, 72 [3d], 168, 336 [2w] hours after reperfusion), rats were sacrificed by decapitation under anesthesia, brain was quickly removed to collect the cerebral cortex. The entire right or left cerebral cortices only were immediately frozen in liquid nitrogen and stored at -70oC until further processing. The right cerebral cortex was also sampled from animals subjected to vascular surgeries without occlusion to serve as sham-operated controls. In some experiments, photography of the whole brain was made for gross visual assessment of the extent of angiogenesis. Total of 39 rats was used in this study. Sample size for each time point in the individual experimental group is further indicated in figure legends. All procedures were approved by our institutional Animal Studies Committee, and were in accordance with the PHS Guide for the Care and Use of Laboratory Animals, USDA Regulations, and the Guidelines of the AVMA Panel on Euthanasia. Sample size for each time point in the individual experimental group is further indicated in figure legends. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques.

Northern Blot Analysis

Total RNA was isolated from frozen brain tissue using the single-step acid guanidinium thiocyanate/ phenol/chloroform extraction method as described before (3). Northern blot analysis has been described previously (1). In brief, RNA samples (15 µg/lane) were applied to 1.2-% agarose gel in the presence of 2.2-M formaldehyde. After electrophoresis, gels were transblotted onto Nytran™ membranes (Gene Screen Plus, DuPont). Membranes were prehybridized at 60°C in a solution containing 1% SDS, 1M NaCl, 10% dextran sulfate and 100 µg/ml of sheared salmon sperm DNA. RT-PCR amplified cDNA probes (10) were labeled with 32P-dCTP using the random-primer labeling method (Amersham, Arlington Heights, IL). Radioactive probes (1×10^6 cpm/ml) were added directly to the prehybridization solution. Following overnight hybridization at 60°C, membranes were washed twice in 2X SSC at room temperature for 5 min each, followed by two 30-min washes at 60°C in 2X SSC/1% SDS and two 30-min washes at 60°C in 0.1X SSC. Membranes were then exposed to Kodak X-Omat/XB-1 films. The radioactive bands in the film were quantified by Densitometer.

Chemicals

All chemicals were of reagent grade purity and

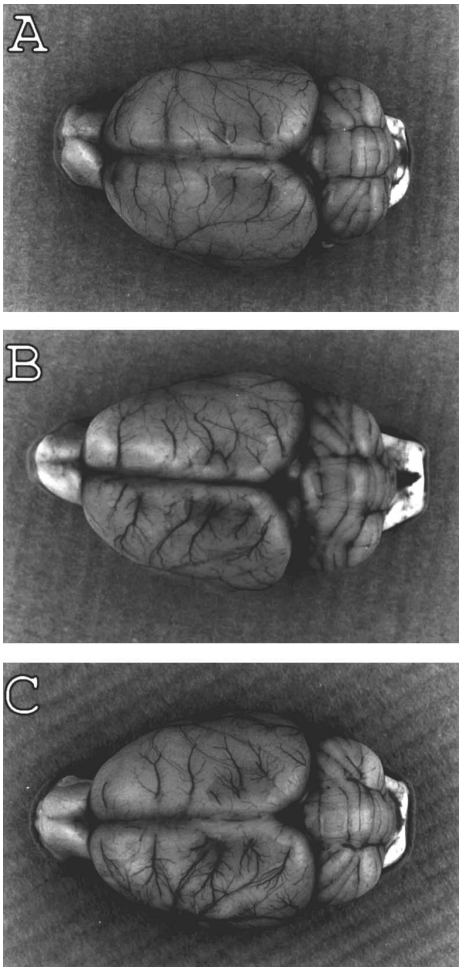

Fig. 1. The effect of focal cerebral ischemia-reperfusion on the vascular density. Rats were subjected to 60-minute ischemia followed by 0-minute (B), and 3 days (C) of reperfusion or no operated normal control (A). The dorsal view of cerebral blood vessels is shown. The upper hemisphere is the control and the lower hemisphere is the ischemic side.

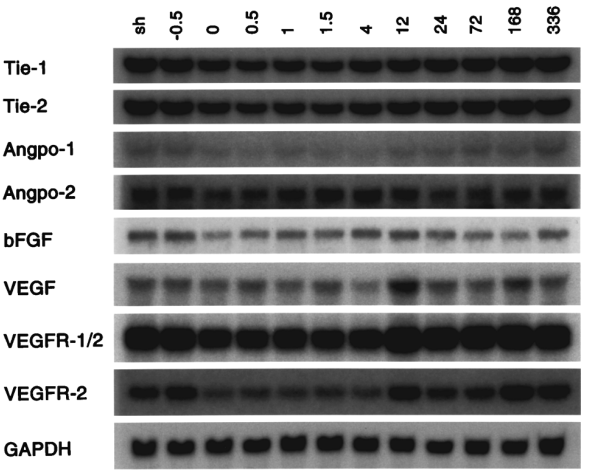

Fig. 2. Representative expression profiles of Angpo-1, Angpo-2, Tie-1, Tie-2, bFGF, VEGF, VEGFR-1/2 and VEGFR-2 mRNA in the contralateral cerebral cortex after transient focal ischemia. Sh denotes sham-operated controls and °V0.5 and 0 represent 30 and 60 min after onset of ischemia or 30 min and 0 min before the initiation of reperfusion respectively. 0.5, 1, 1.5, 4, 12, 24, 72, 168, and 336 denotes 0.5, 1, 1.5, 4, 12, 24, 72, 168, and 336 h of reperfusion following 60-min ischemia, respectively. A representative of triplicate experiments with similar results is shown for each gene.

purchased either from E. Merck (Darmstadt, Denmark) or Sigma Chemical Company (St. Louis, MO) unless otherwise indicated.

Statistics

One-way analysis of variance (ANOVA) was used to compare the temporal expression of mRNAs. The level of significance for differences between groups was further analyzed with post-hoc Fisher's protected t-tests using a statistical software (GB-STAT 5.0.4, Dynamic Microsystem, Inc., Silver Springs, MD). A p value less than 0.05 was considered significant.

Results

Figure 1 shows the dorsal view of cerebral blood vessels at various time points (0 hours and 3 days) after ischemia as compared with a no-operated normal control. Vessel dilation was noted after 60-minute ischemia. Blood vessels were more dilated, with increased vascular density in the ischemic side after 3 days of reperfusion. The temporal expression profiles of Tie-1, Tie-2, Angpo-1, Angpo-2, bFGF, VEGF, VEGFR-1 and VEGFR-2 mRNA in the contralateral cerebral cortex after transient ischemia were examined by Northern blot analysis. Representative expression profiles of each individual gene were shown in Fig. 2 and their quantitative analysis results were shown in Fig. 3. Changes were fewer and smaller in the contralateral side as compare to their induction profiles in the ipsilateral cortex (10). As shown in Fig. 3, results of one-way ANOVA showed that an independent effect was noted for Tie-1 ($p=0.0001$) and VEGFR-2 ($p=0.0168$) mRNA after transient ischemia. The level of Tie-1 mRNA was decreased significantly right after the onset of ischemia and maintained low during the early periods of reperfusion. And then gradually returned to basal level over the late periods of reperfusion. Quantitative analysis shows a 0.5-fold valley decrease as compare to the sham operated control. While the level of VEGFR-2 mRNA was decreased below the basal level during the early periods of reperfusion but increased above the basal level throughout the remaining periods studied, in general.

Although no independent effect was observed in other studied genes. A significant increase in the

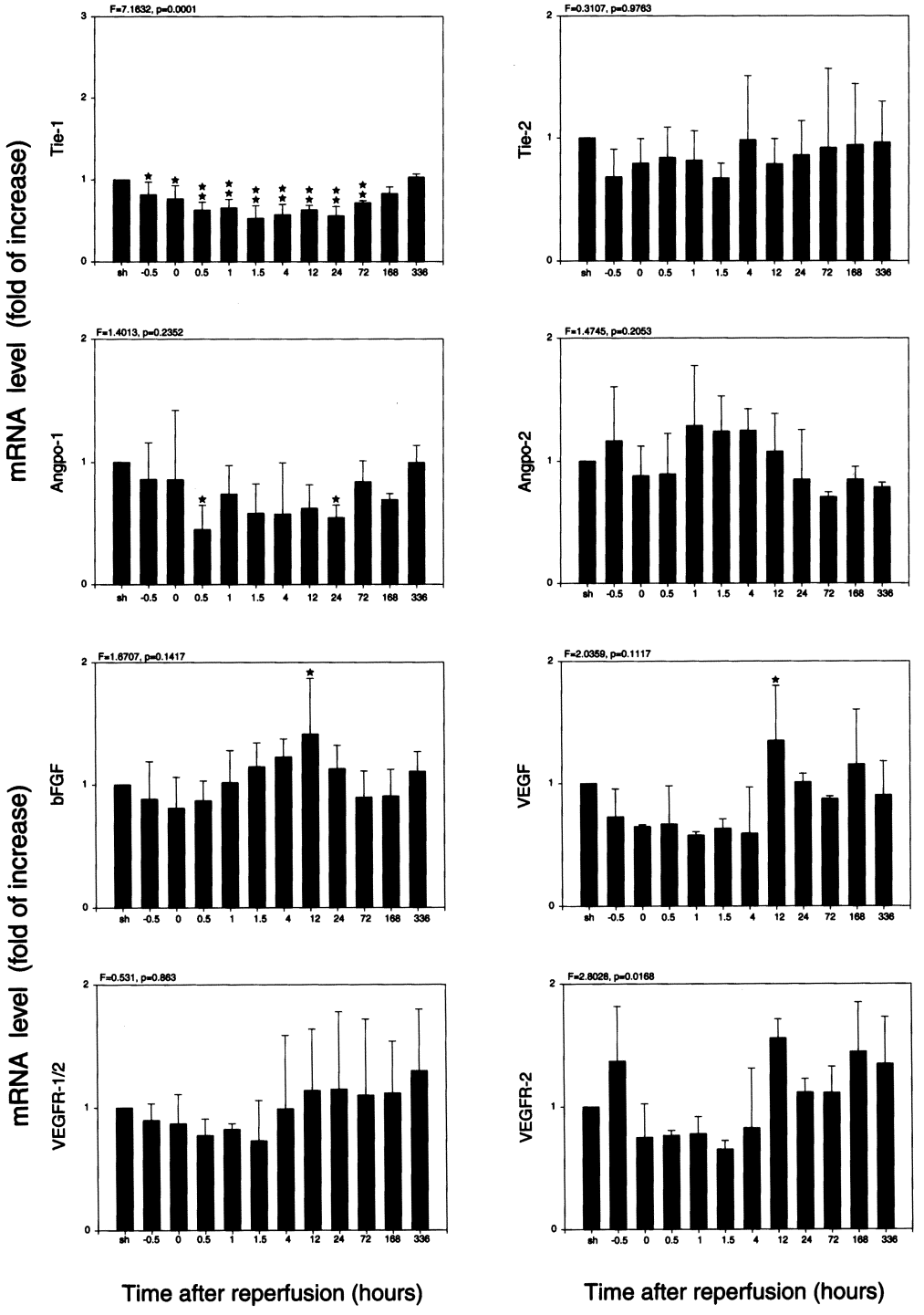

Fig. 3. Time-courses of Angpo-1, Angpo-2, Tie-1, Tie-2, bFGF, VEGF, VEGFR-1/2 and VEGFR-2 mRNA expression in the contralateral cerebral cortex after transient ischemia. The same blots that detected the above genes were subsequently stripped and rehybridized with GAPDH to serve as internal controls. The radioactive bands were quantified and normalized with those derived from GAPDH mRNA. Value obtained from the sham-operated control was arbitrarily defined as 1. Data were mean±SD from 3 animals. ★ and ★★ indicate difference from the sham-operated controls was significant at $p<0.05$ and 0.01, respectively. Please refer to Fig. 2 for more information regarding time after reperfusion.

level of bFGF and VEGF mRNA was noted at 12 hours after the onset of reperfusion as compared to the sham operated control. Furthermore, although not significant, a trend of decrease in the level of Angpo-1 and a trend of increase in the level of Angpo-2 mRNA during the early periods of reperfusion was observed, respectively.

Table 1 shows the Angpo-1/Angpo-2 mRNA

Table 1. Comparison of Angpo-1/Angpo-2 mRNA Ratios in Ipsilateral and Contralateral Cerebral Cortex after Transient Focal Ischemia

	Time after reperfusion (hours)											
	Sh	-0.5	0	0.5	1	1.5	4	12	24	72	168	336
Ipsilateral	1	5	2.23	1.34	1.14	0.89	0.61	0.32	0.16	0.7	1.43	1.66
Contralateral	1	0.73	0.97	0.5	0.57	0.47	0.46	0.58	0.64	1.19	0.81	1.27

P.S. Please refer to Fig.1 for detailed information regarding time after reperfusion. The mean values of Angpo-1 and Angpo-2 were used to obtain this Angpo-1/Angpo-2 mRNA ratio.

ratio, which may be a useful index for the activity of vascular remodeling. In the ipsilateral side, Angpo-1/Angpo-2 mRNA ratio was first dramatically increased (maximal 5-fold) and dropped sharply subsequently (minimal 0.16-fold), and then gradually returned to the basal level thereafter. While in the contralateral side, the Angpo-1/Angpo-2 mRNA ratio was gradually decreased right after the onset of ischemia and early reperfusion periods, then gradually returned to the basal level during the late periods of reperfusion.

Discussions

We have previously reported that, in a rat three-vessel occlusion (MCAO) model, 60 minutes ischemia led to a marked increase in vascular density in the ipsilateral cerebral cortex (9, 10). Although we previously claimed that no morphological (11) and biochemical (1) evidence of ischemic brain injury was noted in the left contralateral cerebral cortex using this model. Subtle changes have been noted in some recent studies, for examples, a 2-fold increased in the 32P-labeling of both PIP and PIP2 were noted in the contralateral cortex after ischemia as compared to those in the contralateral cerebral cortex of sham operated control using this model (8). GFAP mRNA was induced in the contralateral cerebral cortex after transient ischemia using this model (2). And capillary density was also increased in the contralateral cerebral cortex throughout ischemia-reperfusion using this model (Fig. 1; 10); measuring the cerebral blood volume and blood flow with functional MRI further supported this result (data not shown, manuscript in preparation).

There are several possibilities for these subtle changes. Firstly, as we previously reported that with this stroke model, the right MCA cortex sustained severe ischemia with regional blood flow reduction by 88 to 92%. Only very mild ischemia was noted (reduction of blood flow by 10 to 20%) outside the right MCA cortex due to occlusion of both CCAs (1). It is very likely that even only 10 to 20% blood flow reduction is strong enough to challenge the CNS. Secondly, as we previously reported that transient ischemia led to brain edema in the ipsilateral cortex with this model (7, 10), which subsequently increase the intra-cranial pressure and then squeeze the contralateral side. This may introduce stress to cells in the contralateral side. Thirdly, as we previously reported that tissue was liquefied three-week after ischemia with this model (10). It is very likely that certain compensatory mechanism may have been activated in the contralateral side, which increase the energy consumption and blood supply.

Results of Northern blot analysis showed that even only slightly increase in capillary density, significant changes in the level of angiogenesis related genes were observed. In contrast to the ipsilateral cortex, which an independent effect was noted for every studied gene (10), an independent effect was noted only for Tie-1 mRNA and VEGFR-2 mRNA in the contralateral cortex. Furthermore, a $p=0.0001$ was noted for Tie-1 mRNA while a $p=0.0168$ was noted for VEGFR-2 mRNA. It is very likely that both Tie-1 and VEGFR-2 are important in capillary remodeling, but Tie-1 seems much more critical. Interestingly, in contrast to a huge increase in the ipsilateral cortex, Tie-1 mRNA was decreased in the contralateral cortex after transient ischemia. The mechanism and significance for this negative correlation remain to be studied. However, it has been shown that Tie-1 is not required for angioblast differentiation or vessel sprouting during early embryonic development, but is needed during organogenesis to promote angiogenic capillary growth (13, 14). Therefore, maybe the presence and increase in Tie-1 is critical for massive capillary remodeling. Furthermore, in Tie-2 gene knockout mice, although the total number of endothelial cells seems unaffected, the number of large vessels and their caliber are reduced with fewer and straighter branches (13, 14). Interestingly, no increase in Tie-2 mRNA and big vessels were noted in the contralateral cortex after transient ischemia. It is very likely that Tie-2 is important for large vessel remodeling.

Assuming the relative dominance of Angpo-1 and Angpo-2 represents vessel stabilization versus angiogenic sprouting, respectively. Although not significant, there is a trend of decrease in the level of Angpo-1 and a trend of increase in the level of Angpo-2, respectively, in the contralateral cortex after ischemia (Fig. 3). We have previously reported that Angpo-1/Angpo-2 mRNA ratio may be a useful index for the activity of vascular remodeling. Indeed, the degree of change in this index was much smaller in the contralateral cortex (ranging from 0.46 to 1.27) than those in the ipsilateral cortex (ranging from 0.16 to 5; Table 1). Which in lined with the limited increase in capillary density, or low vascular remodeling activity, in the contralateral cortex (Fig. 1). Furthermore, the initial marked increase in this ratio and large vessels seen in the ipsilateral cortex were both missing in the contralateral cortex. It is very likely that increase in the Angpo-1/Angpo-2 mRNA ratio maybe crucial for remodeling into large vessels. Significant increase in bFGF and VEGF mRNA were also noted at 12 hours after reperfusion, however, only around 30% increase as compared to sham operated control, which is much less than their induction in the ipsilateral side (10). The mechanism and significance for this mild induction is not present clear.

In summary, both severe ischemia (ipsilateral cortex) and mild ischemia (contralateral cortex) led to vascular remodeling, and the degree of insult is positively correlated to the vascular remodeling activity. Different induction profiles suggest that these genes may execute their particular functions in different time windows cooperatively and contribute to ischemia-induced vascular remodeling. It is very likely that increase in Angpo-1/Angpo-2 mRNA ratio and Tie-2 may be crucial for remodeling into large vessel. And the presence and increase of Tie-1 maybe important for capillary density increasing. Nevertheless, the detailed mechanisms of induction of angiogenic related genes and whether enhancing and/or prolonging angiogenic sprouting will affect the ischemic outcome remain to be studied.

Acknowledgement

This work was supported by grants from NSC and Academia Sinica, Taipei, Taiwan ROC. We thank Miss Chen J.J. for her technical support.

References

1. An, G., Lin, T.N., Liu, J.S., Xue, J.J., He, Y.Y. and Hsu, C.Y. Expression of c-fos and c-jun family genes after focal cerebral ischemia. *Ann. Neurol.* 33: 57-464, 1993.
2. Cheung, W.M., Wang, C.K., Kuo, J.S. and Lin, T.N. Changes in the level of glial fibrillary acidic protein (GFAP) after mild and severe focal cerebral ischemia. *Chin. J. Physiol*. 42: 227-235, 1999.
3. Chomczynski, P. and Sacchi, N. Single-step of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162: 156-159, 1987.
4. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med*. 1: 27-31, 1995.
5. Kovacs, Z., Ikezaki, K., Samoto, K., Inamura, T. and Fukui, M. VEGF and flt: expression time kinetics in rat brain infarct. *Stroke* 27: 1865-1873, 1996.
6. Krupinski, J., Kaluza, J., Kumar, P., Kumar, S., Path, F.R.C. and Wang, J.M. Role of angiogenesis in-patients with cerebral ischemic stroke. *Stroke* 25: 1794-1798, 1994.
7. Lin, T.N., He, Y.Y., Wu, G., Khan, M. and Hsu, C.Y. Effect of Brain edema on infarct volume in a focal cerebral ischemia model in rat. *Stroke* 24: 117-121, 1993.
8. Lin, T.N., Liu, T.H., Xu, J., Hsu, C.Y. and Sun, G.Y. Brain polyphosphoinositide metabolism during focal ischemia in rat cortex. *Stroke* 22: 495-498, 1991.
9. Lin, T.N., Te, J., Lee, M., Sun, G.Y. and Hsu, C.Y. Induction of basic fibroblast growth factor (bFGF) expression following focal cerebral ischemia. *Mol. Brain Res*. 49: 255-265, 1997.
10. Lin, T.N., Wang, C.K., Cheung, W.M. and Hsu, C.Y. Induction of angiopoietin and tie receptor mRNA expression following cerebral ischemia-reperfusion. *J. Cereb. Blood Flow Metab.* 20: 387-395, 2000.
11. Liu, T.H., Beckman, J.S., Freeman, B.S., Hogan, E.L. and Hsu, C. Y. Polyethylene glycol-conjugated superoxide dismutase and catalase reduce ischemia brain injury. *Am. J. Physiol*. 256: H589-H593, 1989.
12. Plate, K.H. Mechanism of angiogenesis in the brain. *J. Neuropathol. Exp. Neurol.* 58: 313-320, 1999.
13. Puri, M.C., Rossant, J., Alitalo, K., Bernstein, A. and Partanen, J. The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. *EMBO J*. 14: 5884-5891, 1995.
14. Sato, T.N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-MaGuire, M., Gridley, T., Wolburg, H., Risau, W. and Oin, Y. Distinct roles of the receptor tyrosine kinases Tie1 and TIe2 in blood vessel formation. *Nature* 376: 70-74, 1995.
15. Zhang, Z.G., Chopp, M., Lu, D., Wayne, T., Zhang, R.L. and Morris, D. Receptor tyrosine kinase tie 1 mRNA is upregulated on cerebral microvessels after embolic middle cerebral artery occlusion in rat. *Brain Res.* 847: 338-342, 1999.