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Expression of Angiopoietin-1 and the receptor Tie-2 mRNA in rat brains following intracerebral hemorrhage

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Angiopoietin-1 (Ang-1) belongs to a novel family of endothelial growth factors that function as ligands for an endothelial-specific receptor tyrosine kinase (Tie-2). The Ang-1/Tie-2 system may contribute to angiogenesis and vascular remodeling by mediating interactions of endothelial cells with smooth muscle cells and pericytes. The spatial distribution and temporal expression of Ang-1 and Tie-2 in the rat brain were studied following collagenase-induced intracerebral hemorrhage (ICH), by immunohistochemistry and reverse transcription-polymerase chain (RT-PCR) analysis, respectively. Immunohistochemical analysis revealed that some Ang-1 or Tie-2-positive dilated vessels resided around the hematoma and extended into the clot. RT-PCR analysis showed that Ang-1 and Tie-2 mRNA signal was detected at 2 days and persisted for 28 days after ICH. These findings suggest that ICH could lead to upregulation of Ang-1 and the receptor Tie-2 mRNA.

Key words: intracerebral hemorrhage, angiopoietin-1, Tie-2, receptor, angiogenesis

INTRODUCTION

Intracerebral hemorrhage (ICH) is a common and often fatal stroke subtype. Primary ICH accounts for approximately 8–14% of all strokes in Western countries (Brown et al. 1996), and a considerably higher proportion in populations of China, Korea, and Japan (Lo et al. 1994, Yang et al. 2004). Indeed, the 30-day mortality rate is about 50%, and neurological recovery in survivors is often poor (Giroud et al. 1991, Fogelholm et al. 1992, Broderick et al. 1994). However, currently there is no medical therapy available for these patients except for blood pressure reduction and neurosurgical evacuation of the hematoma (Mendelow et al. 2005, Qureshi et al. 2005).

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Angiogenesis, which refers to the expansion or remodeling of pre-existing blood vessels (Ward and Dumont 2002), is a fundamental requirement for organ development and differentiation during embryogenesis as well as for wound healing and reproductive functions in the adult (Folkman 1995). The molecular events governing angiogenesis are complex and involve multiple families of proteins and receptors, including the vascular endothelial growth factor (VEGF) family (Veikkola and Alitalo 1999) and the angiopoietin (Ang) family (Ward and Dumont 2002). In contrast to VEGF, which stimulates endothelial cell migration and promotes cell survival and proliferation, Ang-1 does not appear to stimulate cell proliferation (Davis et al. 1996) but does play a crucial role in mediating reciprocal interactions between the endothelium and surrounding matrix and in causing maturation and stabilization of vessels (Suri et al. 1996).

Recent studies have proved that in the central nervous system Ang-1 and endothelial-specific receptor tyrosine kinase Tie-2 were observed to be involved in a wide variety of disorders, ranging from vascular malformations and brain tumors to cerebral ischemia (Zhang 2002, Harrigan 2003, Nourhaghighi et al. 2003, Ardelt et al. 2005, Hohenstein et al. 2005, Ward et al. 2007). However, so far there have been no reports about the expression of Ang-1/Tie-2 following ICH. Accordingly, the purpose of our present work is to evaluate whether the expression of Ang-1 and its receptor Tie-2 is altered in rat brains with collagenase-induced ICH, which might widen our understanding in mechanisms of rehabilitation after the stroke.

METHODS

Animal preparation

Studies were carried out on adult male Sprague-Dawley rats (250–300 g, 8–10 weeks of age) obtained from the Experimental Animal Science Center of Central South University, which were housed under identical conditions (room temperature 25°C, 12 h light-dark cycle) and allowed free access to food and water. The experimental protocol was performed under compliance with guidelines of Central South University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). Rats were randomly assigned to sham-operated control group (n=8, per time point) and ICH group (n=8, per time point).

Collagenase-induced intracerebral hemorrhage

The intracerebral hemorrhage was induced according to Rosenberg and coauthors (1990). After fasting for a night, animals were anesthetized with chloral hydrate (400 mg/kg) intraperitoneally, then fixed on a stereotactic frame (STOELTING) pronely. Following a scalp incision, a small cranial burr was drilled near the right coronal suture 3.2 mm lateral to the midline. 0.5 U bacterial collagenase (type VII, Sigma) in 2.5 μl 0.9% sterile saline was injected into right globus pallidus (1.4 mm posterior and 3.2 mm lateral to bregma, 5.6 mm ventral to cortical surface) with a 5-μl Hamilton syringe for over 5 minutes, with the needle left there for 5 minutes afterwards. The bone hole was sealed with bone wax allowing for subsequent intracranial pressure elevations due to the hemorrhage, the scalp wound was sutured, and each animal was placed in a warm box to recover. For the sham group, 2.5 μl 0.9% sterile saline instead of the collagenase in saline was injected to the same site. During the procedure, rectum temperature was monitored and maintained at 37.5°C with a feedback controlled heating pad.

Specimen preparation

The randomly chosen animals from the two groups were deeply anesthetized with chloral hydrate (800 mg/kg) at 2 days, 4 days, 7 days, 14 days, 21days, 28 days postoperation. For histological assessment, animals (n=3, per time point) were transcardially perfused with 0.9% saline followed by 250 ml ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4). The removed brains were post-fixed in the same fixative for 2 hours, then transferred to 20%, 30% sucrose in 0.1 M phosphate buffer (pH=7.4) sequentially at 4°C until sinking. Brains were cut for 30 μm coronal sections at −20°C with a cryostat (Leica CM1900, Germany), some of which were collected in 0.01 M phosphate buffer saline (pH=7.4) and stored at 4°C. For reverse transcription-polymerase chain reaction (RT-PCR), rats (n=5, per time point) were killed by decapitation, the brains were immediately removed and the tissues in striatum adjacent to the hematoma and without the needle track were dissected and frozen at −196°C in liquid nitrogen.

Immunohistochemistry

Sections were brought to room temperature, and incubated in 3% H_2O_2 in methanol for 15 minutes. After washing three times in PBS for 5 minutes each, nonspecific binding was blocked in 5% bovine serum albumin (BSA, Sigma. USA) for 1 hour at 37°C. Sections were not washed, incubated with goat anti-Ang-1 (1:70, Santa Cruz Biotech, USA) or goat anti-Tie-2 (1:70, Santa Cruz Biotech, USA) overnight at 4°C, then with a biotinylated anti-goat IgG (1:100) for 1 hour. Color development was performed with a Vectastain ABC kit (Vector Laboratories, USA) according to the vendor´s protocol. For negative control, 1% BSA was used instead of the primary antibody.

Reverse Transcription-Polymerase Chain Reaction RT-PCR

RNA and cDNA preparation

Total RNA from the border of the hematoma was extracted using TRIzol Reagent following the manufacturer's instructions (Invitrogen, USA). RNA was resuspended in diethypyrocarbonate-treated water. Samples were then extracted with phenol-choroform and precipitated with ethanol. Reverse transcription was performed on 2 μg of total RNA using 1 μg/μl oligo$(dT)_{18}$ (1 μl), 10 mM dNTPMix (2 μl), RNase inhibitor (1 μl) and 200 u/μl M-MuLV-Reverse Transcriptase (1 μl) at 70°C for 5 minutes, at 37°C for 5 minutes, at 42°C for 60 minutes and at 70°C for 10 minutes consecutively following the manufacturer's instructions (Fermentas, CA) and then, cDNA was stored at −20°C.

Polymerase chain reaction

Polymerase chain reaction primer sequences were chosen from published document as listed in Table I (Lin et al. 2000). In brief, polymerase chain reaction was carried out in a final volume of 25 μl containing 2 μl cDNA, 2.5 μl 10× PCR buffer, 0.5 μl rTaq, 2 μl of each primer and 16 μl diethypyrocarbonate-treated water, the mixture was incubated in a thermal cycler (Tenchne TC-512, UK) for 31 cycles using the following profile: 94°C for 7 minutes, then repeat cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 90 seconds. Samples were then incubated at 72°C for 7 minutes and cooled to 4°C. Each set of PCR reactions included control samples run without RNA or in which the RT step was omitted to ensure that PCR products resulted from amplification from the analyzed mRNA rather than genomic ^{p}DNA. In the experiment we also used rat β-actin as an internal standard. Polymerase chain reaction products were electrophoresed using 1× TBE buffer on 2% agarose gel containing ethidium bromide. Semi-quantitation of bands was accomplished by using a computer-assisted Tanon GIS-2020 Analysis System (Tanon Co. China). Measurements were normalized to the optical density of the β-actin band.

Statistical analysis

Data were expressed as means ± standard deviation (SD). Analysis of variance (ANOVA) followed by Student-Newman-Keuls' *post-hoc* test was used to compare means.

RESULTS

The spatial distribution of Ang-1 and Tie-2 after ICH

To examine in which brain areas the expression of Ang-1 and Tie-2 was changed, immunohistochemistry was used. The immunopositive vessels could be observed only occasionally in sham-control rats, as well as after ICH in the hemisphere contralateral to the injury, or in the ipsilateral cortex to the clot. Some Ang-1 or Tie-2 positive dilated vessels appeared at basal ganglion around the hematoma from 2 days to 7 days after ICH induction; thereafter, positive vessels extended into the clot (Fig. 1). The observation was obtained from the 3 animals of either group at each time point.

Table I

Gene		primer sequence (5'-3')	product (bp)
Ang-1	Forward	GAAAATTATACTCAGTGGCTGGAAAAA	330
	Reverse	TTCTAGGATTTTATGCTCTAATAAACT	
Tie-2	Forward	ATTGACGTGAAGATCAAGAATGCCACC	375
	Reverse	ATCCGGATTGTTTTTGGCCTTCCTGTT	
β-actin	Forward	CGTTGACATCCGTAAAGAC	201
	Reverse	TGGAAGGTGGACAGTGAG	

Primers for Reverse Transcription-Polymerase Chain Reaction

RT-PCR assay of Ang-1 and Tie-2 mRNA

As it is demonstrated by immunohistochemistry that the expression of Ang-1 and Tie-2 was changed predominantly around the hematoma, we evaluated the alteration of Ang-1 and Tie-2 mRNA at the basal ganglion ipsilateral to ICH by RT-PCR. In sham-operated rats, no Ang-1 or Tie-2 mRNA signal was detected. However, Ang-1 and Tie-2 mRNA signals could be detected at 2 days after ICH, then the levels continued to increase notably from 7 days till 28 days (Fig. 2).

DISCUSSION

Intracerebral hemorrhage (ICH) may lead to severe reduction of blood supply and hypoxia in the affected region resulting from the compression by hematoma (Klein et al. 1986, Mendelow 1993, Belayev et al. 2003, Kim-Han et al. 2006). Studies have proved that hypoxia may induce angiogenesis (Kaur et al. 2005), and our recent observations have also demonstrated that ICH could induce angiogenesis in the perihematomal tissue, and that the new-born vessels extended into the clot approximately after 7 days (Tang et al. 2007). In the present study, histological observation showed that Ang-1 and Tie-2 was primarily expressed in enlarged vessels after the stroke, and that the positive vessels appeared in the hematoma after 14 days. Since it was reported by several groups that Ang-1 and Tie-2 could mediate angiogenesis in ischemic stroke (Lin et al. 2000, Harrigan 2003), our data suggest that Ang-1 and Tie-2 might also have a close relationship with angiogenesis in the rat brains following ICH. Moreover, it was reported also that nitric

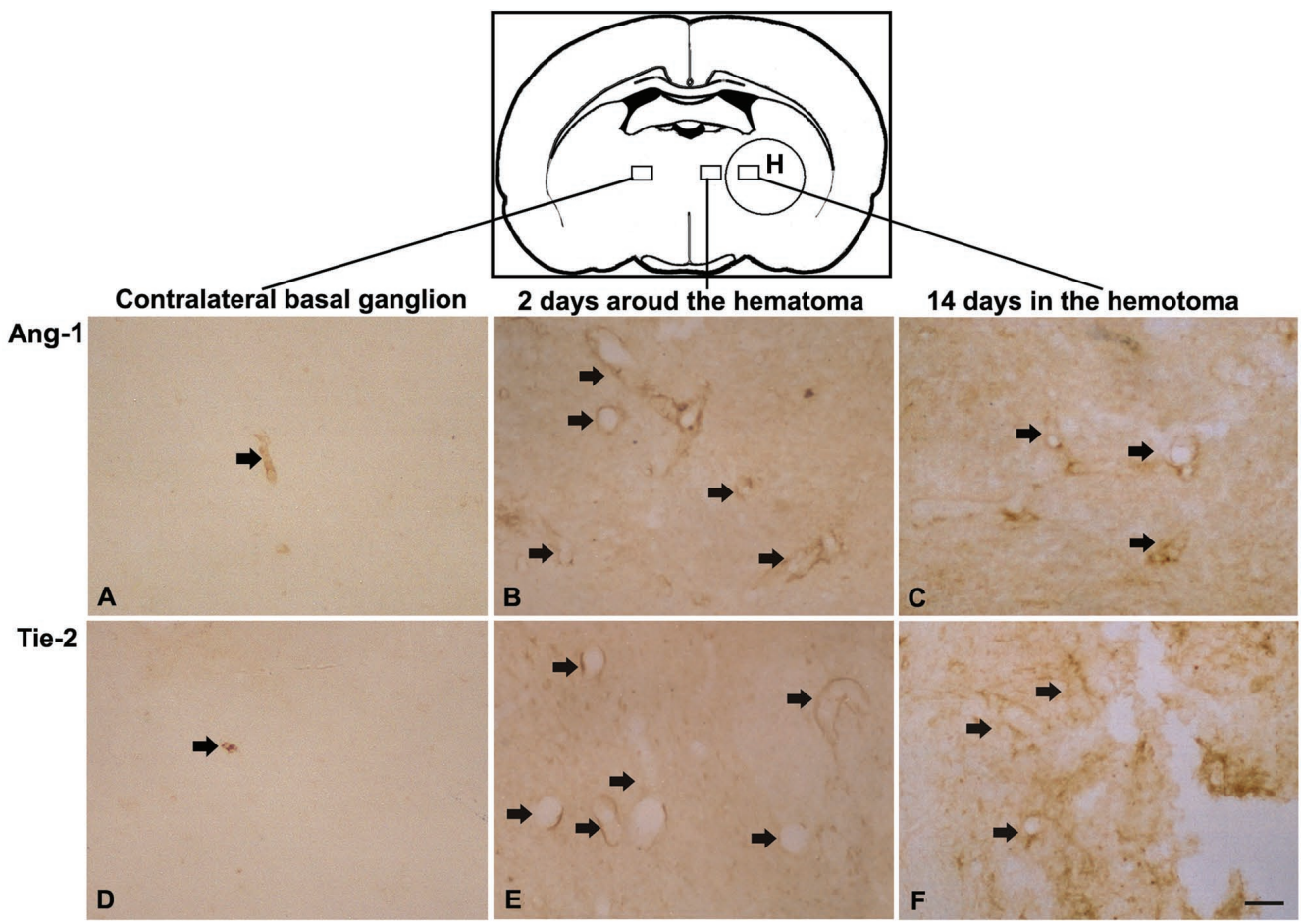

Fig. 1. The spatial distribution of Ang-1 and Tie-2 after ICH. After ICH induction, few Ang-1(A) or Tie-2 (D) positive slim vessels (arrow) could be observed at the basal ganglion contralateral to the clot, but some Ang-1(B) or Tie-2 (E) positive dilated vessels (arrow) appeared around the hemotoma at 2 days, and the vessels (arrow) extended into the clot at 14 days (C, F). The top panel: schema of hemorrhagic region and areas where the pictures were taken. (H) hemorrhage. Bar is 100 μm.

oxide (NO), an important endothelium-derived relaxing factor, was over-produced after ICH (Peng et al. 1997). The factor could not only dilate the vessels, but also mediate proliferation of endothelial cells by increasing expression of VEGF and Ang-1/Tie-2 after ischemic stroke (Jorens et al. 1993, Zhang et al. 2003, Zacharek et al. 2006). Hence, NO may cause the substantial change in the lumen diameter of the positive vessels and alter expression of Ang-1/Tie-2 mRNA following ICH.

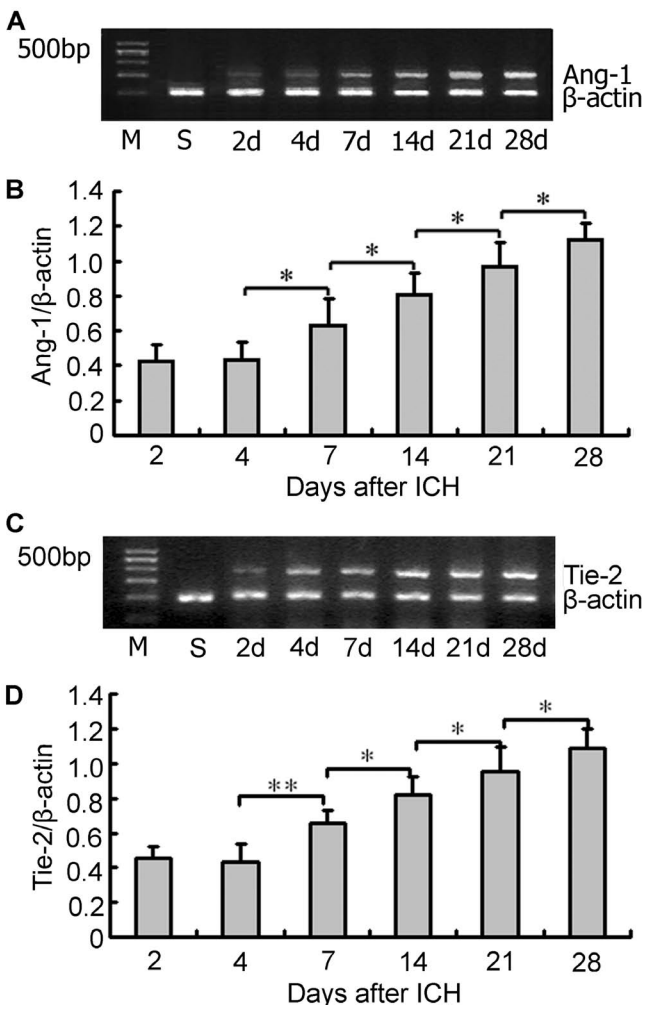

Fig. 2. Representative expression profile of Ang-1 (A) and Tie-2 mRNA (C) and alteration of Ang-1 (B) and Tie-2 mRNA (D) concentrations at ipsilateral basal ganglion after ICH. (S) sham operated animal. X-axis indicates postoperative time in day (d). In sham-operated rats, no Ang-1 or Tie-2 mRNA signal was detected. After ICH induction, both Ang-1 and Tie-2mRNA signals were first detected at 2 days. Notable increases in Ang-1 and Tie-2 mRNA could be detected in the border of hematoma from 7 days and persisted for 28 days. Data are means ± SD, n=5; * P<0.05, ** P<0.01.

The expression of Ang-1 and Tie-2 was reported to be very weak in the adult rat brains, which might play a role in maintaining the integrity of the adult vasculature (Dumont et al. 1992, Thurston et al. 1999, Thurston et al. 2000). The result of the present study which showed lack of Ang-1 or Tie-2 mRNA signal in sham-operated rats is in line with those observations. However, we found that Ang-1 and Tie-2 mRNA is upregulated following ICH. In the early stage of ICH, hypoxia may partly contribute to the upregulation of Ang-1 and Tie-2 mRNA (Park et al. 2003). ICH could also induce expression of inflammatory cytokines, such as TNF-α, IL-1β and ICAM-1, which mainly increased in the early phase (Gong et al. 2000, Hua et al. 2006, Wu et al. 2006). Numerous studies have shown that a high dose of inflammatory cytokines could lead to the down-regulation of Ang-1 and Tie-2 mRNA, while a low dose of inflammatory cytokines could induce the overexpression of Ang-1 and Tie-2 mRNA (Fan et al. 2004, Scott et al. 2005, Hangai et al. 2006). Taken together, it could explain why the expression of Ang-1 and Tie-2 mRNA was relatively weak at 2–4 days and then increased gradually in the current study.

Although Ang-1 has little effect in the early phase of angiogenesis, the low level of Ang-1mRNA at the onset of ICH in the present study may reflect an initial attempt of the affected region to counteract inflammation and stabilize the vascular integrity (Gamble et al. 2000). *In vitro* experiments demonstrated that Ang-1 has specific effects on endothelial cells, which potently induces sprouting, chemotactic response (Koblizek et al. 1998), network formation (Witzenbichler et al. 1998), and survival in apoptosis (Kwak et al. 1999, Papapetropoulos et al. 1999). Furthermore, the phenotypic analysis of Ang-1 knockout animals shows a decrease in the amount and complexity of capillary branches while overexpression of Ang-1 increases the number and branching complexity of vessels (Hayes et al. 1999), which suggested that Ang-1 was crucial for complexity of capillary branches. In addition, Ang-1 could mediate remodeling and stabilization of endothelial cells and extracellular matrix interactions and play a role in the recruitment of peri-endothelial mesenchymal cell to the vessels (Suri et al. 1996). It has been revealed that the mRNA expression of Ang-2 exceeded that of Ang-1 within the neomembranes of chronic subdural hematoma, which was accompanied by ongoing angiogenesis including destabilization of the structure of existing blood vessels, endothelial cell prolif-

eration, and tube formation as observed in the membrane (Hohenstein et al. 2005). Hence, in the present study the Ang-1 mRNA expression in a persistently rising pattern might suggest the angiogenic switch from new vessel formation to vessel maturation, and the late notable upregulation of Ang-1 could elucidate why the positive vessels could extend into the clot and persist for 28 days without being resolved.

Tie-2, the receptor of Ang-1, is expressed primarily on endothelial cells (Dunont et al. 1992). And it was demonstrated that Tie-2 might be involved in a physiological regulation of flow shear stress-dependent endothelial activity (Tai et al. 2005), which suggested that such a low level of Tie-2 in the intact brain might be enough to accomplish the functions. Disruption of murine Tie-2 function in transgenic mice resulted in early embryonic lethality secondary to distinct defects in microvascular development, characterized by reduced endothelial cell number, abnormal vascular branching, and compromised endothelial integrity (Dumont et al. 1994, Sato et al. 1995). These findings indicated that Tie-2 function was not required for the earliest stages of endothelial differentiation and vascular patterning but was crucial for the formation of the microvasculature during embryonic angiogenesis. The results of our study which show that ICH could also lead to a prolonged increase in Tie-2 mRNA, coinciding with the induction of its ligand Ang-1, are consistent with the role for Tie-2 during angiogenesis in adult tissues. Importantly, Tie-2 plays a vital role in the mature vasculature as well, when phosphorylated by Ang-1, and disruption of Tie-2 signaling *via* Ang-1 knockout, Tie-2 knockout, or a Tie-2 dominant-negative approach all yielded similar phenotypes (Dumont et al. 1994, Sato et al. 1995, Suri et al. 1996). Therefore, our data suggested that Tie-2 was required for the stabilization and maturation of the newly-formed vessels in ICH.

CONCLUSION

In summary, our study demonstrated for the first time that ICH could lead to the upregulation of Ang-1 and its receptor Tie-2 mRNA. Angiogenesis is a complex process which needs orchestrated effects of many growth factors, and Ang-1 is another important factor for angiogenesis besides VEGF. Therefore, we presume that the Ang-1/Tie-2 system may also play a crucial role in the regeneration of microvessels after intracerebral hemorrhage.

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