Differential Regulation of Thrombospondin-1 and Thrombospondin-2 After Focal Cerebral Ischemia/Reperfusion

Teng-nan Lin, PhD; Gyoeng-Moon Kim, MD, PhD; Jean-Ju Chen, BS; Wai-Mui Cheung, BS; Yong Y. He, MD; Chung Y. Hsu, MD, PhD

Background and Purpose—Angiogenesis occurs after cerebral ischemia, and the extent of angiogenesis has been correlated with survival in stroke patients. However, postischemic angiogenesis is short-lived and may be completely terminated within a few weeks after ischemic insult. The molecular mechanism underlying the dissolution of postischemic angiogenic processes is poorly understood. Although the expression of angiogenic genes has been studied in ischemic stroke models, the activation of angiostatic genes after cerebral ischemia has not been investigated. Thrombospondin (TSP)-1 and TSP-2 are naturally occurring angiostatic factors, which inhibit angiogenesis in vivo. The aim of the present study was to explore the expression of TSP-1 and TSP-2 in relation to the evolution of angiogenic process in a focal ischemia model in rats.

Methods—Rats underwent cortical ischemia in the middle cerebral artery territory for 60 minutes and reperfusion for up to 2 weeks. Northern and Western blot analysis were used to study the temporal profile of TSP-1 and TSP-2 expression at the mRNA and protein level, respectively. In situ hybridization and immunohistochemical studies were used to examine the spatial expression patterns. Double immunostaining was applied to define the cellular origins of TSP-1 and TSP-2.

Results—A biphasic expression of TSP-1 was noted after ischemia, peaking at 1 and 72 hours. Endothelial cells in the leptomeninges were the only source of the first TSP-1 peak, whereas endothelial, glial, neuronal, and macrophage cells contributed to the second peak of TSP-1 expression. TSP-2 expression occurred much later and in a monophasic manner, peaking 2 weeks after ischemia. TSP-2 immunoreactivity was observed in endothelial, neuronal, and macrophage, but not glial, cells. TSP-1 was expressed before the peak of angiogenesis, whereas robust TSP-2 expression occurred at the peak of angiogenesis and continued into the period when angiogenesis had completely resolved.

Conclusions—Robust expression of TSP-1 and TSP-2, 2 major angiostatic factors, was noted in the ischemic brain with different temporal expression profiles from different cellular origins. The expression of these angiostatic factors, especially TSP-2, likely contributes to the spontaneous resolution of postischemic angiogenesis. Further studies are needed to explore the molecular mechanisms that regulate the balance of angiogenic and angiostatic factors in the ischemic brain. (Stroke. 2003;34:177-186.)

Key Words: angiogenesis factor ■ angiogenesis inhibitors ■ endothelium ■ gene expression ■ stroke, ischemic ■ rats

Ischemic brain injury is a consequence of a 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. The extent of angiogenesis has been correlated with survival in stroke patients.1,2 Despite the postischemic induction of many angiogenic factors (eg, vascular endothelial growth factor [VEGF], basic fibroblast growth factor, and angiopoietin), we observed eventual regression of this postischemic angiogenic sprouting.3–5 Because angiogenic activity reflects a balance between the angiogenic and angiostatic drives,6–11 it is expected that the expression of angiostatic factors may contribute to the resolution of postischemic angiogenesis.

Among major angiostatic factors, thrombospondin (TSP)-1 and TSP-2 have gained increasing importance in recent studies. TSP-1 and TSP-2 belong to a family of secreted, multidomain, and multimeric extracellular glycoproteins that participate in cell-to-cell and cell-to-matrix communication.12 There are 5 family members, each representing a separate gene product, in most vertebrate species. Each of these 5 gene
products has a specific pattern of expression in embryonic and adult tissues.13,14 The TSP gene family is divided into 2 subfamilies, A and B, according to their overall molecular organization. The subgroup A proteins, TSP-1 and TSP-2, are assembled as trimers. Each subunit of these trimeric molecules is composed of an N-terminal heparin-binding domain, a linker domain enclosing 2 cysteine residues required for trimerization, a procollagen-homology domain with 3 properdin-like type I repeats, 3 epidermal growth factor–like type II repeats, 7 calcium-binding type III repeats, and a globular C-terminal domain.15 The subgroup B proteins, TSP-3, TSP-4, and TSP-5/COMP, are assembled as pentamers, and each subunit contains similar domains found in subgroup A, except that there are 4 instead of 3 type II repeats but no procollagen-homology domain or 3 type I repeats.16 It is the type I repeats that make TSP-1 and TSP-2 naturally occurring inhibitors of angiogenesis, capable of inducing apoptotic endothelial cell death.14,17,18

During embryonic development in mice, TSP-1 mRNA is expressed in the capillaries, and TSP-2 mRNA is expressed in the capillaries and large vessels. Despite their structural similarity and partially overlapping sites of expression during development, the essential functions of TSP-1 and TSP-2 are distinct and not redundant, and each has its characteristic temporal and spatial expression profile.19 However, it is clear that TSP-1 and TSP-2 are angiostatic. Both TSP-1–and TSP-2–null mice showed increased vascularity in skin wounds.20 To our knowledge, TSP-1 and TSP-2 expression in the ischemic brain has not been studied previously. The present study was undertaken to explore whether the expression of TSP-1 and TSP-2 is altered after focal cerebral ischemia/reperfusion.

**Materials and Methods**

**Materials**
All chemicals and reagents were purchased from E. Merck or Sigma Chemical Co, unless specified.

**Stroke Model**
The focal cerebral ischemia/reperfusion model in rats has been described previously.21,22 In brief, male Long-Evans rats weighing 250 to 300 g were anesthetized with chloral hydrate (360 mg/kg body wt IP). 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 the ligation was confirmed by microscope. Both common carotid arteries were then occluded by using nontraumatic aneurysm clips. After 60-minute ischemia, the aneurysm clips and the suture were removed, and restoration of blood flow in all 3 arteries was verified. While the animals were under anesthesia, the rectal temperature was monitored and maintained at 37.0 ± 0.5°C by using a homeothermic blanket (Harvard Instruments). After the ischemic insult, the 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 laboratory Chow ad libitum until the end of the experiments. At the end of each experiment (30 and 60 minutes after the onset of ischemia or 30, 60, and 90 minutes, 4 and 12 hours, 1 and 3 days, and 1 and 2 weeks after reperfusion), rats were killed by decapitation under anesthesia, and the brains were quickly removed to collect the cerebral cortex. The ischemic right and the uninjured left MCA cortices were separated and frozen immediately in liquid nitrogen and stored at −70°C until further processing. In some experiments, the animals were euthanized by transcardial perfusion with normal saline under anesthesia, followed by cold 4% paraformaldehyde. The whole brains were removed and cryoprotected in 30% sucrose at 4°C overnight. Animals subjected to vascular surgeries without right MCA or bilateral common carotid artery occlusion served as sham-operated controls. Arterial blood gases, mean arterial pressure, and heart rate were also monitored in selected animals before and during ischemia and for 30 minutes after the initiation of reperfusion. The values were within normal range before, during, and after ischemia. The regional cerebral blood flow in this model was also studied with the use of [14C]iodoantipyrine. In this stroke model, only the right MCA cortex sustained severe ischemia, with regional blood flow reduction of 88% to 92% (n = 4). Only very mild ischemia was noted (reduction of blood flow of 10% to 20%) outside the right MCA cortex.23 The left MCA cortex, which sustained no ischemic injury after transient occlusion of the right MCA and bilateral common carotid arteries, was also collected to serve as another set of controls. All the procedures were approved by the institutional animal studies committees and were in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Public Health Service, US Department of Agriculture Regulations, and the Guidelines of the American Veterinary Medical Association Panel on Euthanasia.

**DNA Isolation and Reverse Transcription**
Total RNA was isolated from the frozen cerebral cortex by using the single-step acid guanidinium thiocyanate–phenol–chloroform extraction method, as previously described.4 Briefly, total RNA (4 μg) was incubated with 200 U of MMLV reverse transcriptase (RTase, Clontech) in a buffer containing a final concentration of 50 mmol/L Tris–Cl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl2, 20 U RNase inhibitor, 1 μmol/L poly(dT) oligomer, and 0.5 mmol/L of each dNTP in a final volume of 20 μL. The reaction mixture was incubated at 42°C for 1 hour and then at 94°C for 5 minutes to inactivate the enzyme. A total of 80 μL diethyl pyrocarbonate–treated water was added to the reaction mixture before storage at −70°C.

**Polymerase Chain Reaction**
Polymerase chain reaction (PCR) primers for TSP-1 and TSP-2 were chosen from sequences in the GenBank: TSP-1, forward, 5′-CTC ACC TAT GCT CAG GCT-3′, and TSP-1, reverse, 5′-GCA TTC CAG AGT CTG GC-3′ (410 bp); TSP-2, forward, 5′-CTG TGT CAA CAC AGC CTG GC-3′, and TSP-2, reverse, 5′-TCC TTC TCA TCG GTC ACA CCG-3′ (390 bp). Five microliters of the final reverse transcription (RT) reaction solution was used in the PCR reaction. PCR was carried out in a final volume of 50 μL containing 200 μmol/L each of dATP, dCTP, dGTP, and dTTP, 5 pmol of each primer, 1.25 U Tag polymerase (BRL), 20 mmol/L Tris–Cl (pH 8.4), 1.5 mmol/L MgCl2, and 50 mmol/L KCl. The mixture was incubated in a thermal controller for 35 cycles by using the following profile: (1) 94°C for 5 minutes, (2) repeat cycles at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 2 minutes, and (3) 72°C for 10 minutes (GeneAmp 2400, PE). PCR products were resolved on 2% agarose gel for DNA fragment size verification, eluted, and subcloned (Invitrogen) for sequence identity and then served as a probe to detect TSP-1 and TSP-2 mRNA in Northern blot analysis and in situ hybridization.

**Northern Blot Analysis**
Northern blot analysis has been described previously.4 Briefly, RNA samples (15 μg per lane) were applied to 1.2% agarose gel in the presence of 2.2 mol/L 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, 1 mol/L NaCl, 10% dextran sulfate, and 100 μg/mL sheared salmon sperm DNA. RT–PCR–amplified TSP-1, TSP-2, and GAPDH cDNA probes were labeled with [32P]dCTP by using the random-primer labeling method (Amersham). Radioactive probes (1 × 106 cpm/mL) were added directly to the prehybridization solution. After overnight hybridization at 60°C, membranes were
washed twice in 2× SSC at room temperature for 5 minutes each, followed by two 30-minute washes at 60°C in 2× SSC/1% SDS and two 30-minute washes at 60°C in 0.1× SSC. Membranes were then exposed to Kodak X-Omat/XB-1 films. The radioactive bands were quantified by a densitometer.

**In Situ Hybridization**

In situ hybridization to detect the regional distribution of mRNA signals has been described previously. In brief, brain slices were frozen-sectioned at 25-μm thickness and mounted on poly-L-lysine–coated slides. Brain sections were subjected to 0.001% proteinase K digestion at 37°C for 30 minutes, then immersed in 0.1 mol/L triethanolamine containing 0.25% acetic acid anhydride at room temperature for 10 minutes, and subsequently dehydrated in 50%, 70%, 95%, and 100% ethanol (3 minutes each). Hybridization was carried out in a solution containing 12.5 mol/L formamide, 10% dextran sulfate, 0.3 mol/L NaCl, 1× Denhardt’s solution, 10 mmol/L Tris-Cl (pH 8.0), 500 μg/mL sheared salmon sperm DNA, 100 μg/mL tRNA, 20 mmol/L dithiothreitol, and 107 cpm/mL of probes at 55°C. RT-PCR–amplified cDNA probes were labeled with [33P]dCTP by using the random-primer labeling method (Amer-Sham). After overnight hybridization, slides were then washed sequentially in 2×, 1×, 0.2×, and 0.1× SSC at 55°C for 30 minutes each, followed by dehydration in 50%, 70%, 95%, and 100% ethanol for 3 minutes each. Brain sections were exposed to Kodak BioMax-MR-1 film. In control experiments, sections were incubated with a 100-fold excess of unlabeled probe or pretreated with RNase A (100 μg/mL, 37°C, 30 minutes). These experiments resulted in no or negligible signals.

**Western Blot Analysis**

The right (ischemic) and left MCA cortices were separated at various time points after ischemia/reperfusion and homogenized in a buffer containing 10 mmol/L HEPES, 1.5 mmol/L MgCl2, 10 mmol/L KCl, 500 μg/mL sheared salmon sperm DNA, 100 μg/mL tRNA, 20 mmol/L dithiothreitol, and 107 cpm/mL of probes at 55°C. RT-PCR–amplified cDNA probes were labeled with [33P]dCTP by using the random-primer labeling method (Amer-Sham). After overnight hybridization, slides were then washed sequentially in 2×, 1×, 0.2×, and 0.1× SSC at 55°C for 30 minutes each, followed by dehydration in 50%, 70%, 95%, and 100% ethanol for 3 minutes each. Brain sections were exposed to Kodak BioMax-MR-1 film. In control experiments, sections were incubated with a 100-fold excess of unlabeled probe or pretreated with RNase A (100 μg/mL, 37°C, 30 minutes). These experiments resulted in no or negligible signals.
0.5 mmol/L dithiothreitol, 1% NP-40, 1 μg/mL leupeptin, and 1 μg/mL aprotinin (pH 7.9) and then centrifuged at 14 000g. Proteins (40 μg) from the supernatant of each sample were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes by electrophoresis. The membranes were blocked in TBST buffer containing 20 mmol/L Tris-HCl, 5% nonfat milk, 150 mmol/L NaCl, and 0.05% Tween 20 (pH 7.5) for 1 hour at room temperature. The blots were incubated with either a primary monoclonal mouse anti–TSP-1 antibody (1:250, BD Transduction Laboratories), a mouse anti–TSP-2 antibody (1:250, BD Transduction Laboratories), or a mouse anti-actin antibody (1:500, BD Transduction Laboratories), followed by a secondary alkaline phosphatase–conjugated anti-rabbit IgG antibody (1:5000, Promega). The Western blots were visualized with the Blot AP System (Promega).

**Immunohistochemical Staining**

Immunohistochemical methods to study the regional distribution of gene products have been described previously.24 Briefly, brain slices of 25-μm thickness were permeabilized with 0.3% Triton X-100 and 4% normal goat serum in 0.01 mol/L PBS (pH 7.4) for 20 minutes.

**Figure 2.** In situ hybridization for spatial expression of TSP-1 and TSP-2 mRNA. Sixty-minute, 1-day, and 7-day reperfusion after 60-minute ischemia is shown; sham denotes sham-operated control. Brain slices (25 μm) were hybridized with 32P-labeled probes, as described in the text. Note the increase in signal intensity in both TSP-1 and TSP-2 mRNA in the ischemic right MCA cortex after ischemic insult. No obvious changes in signal intensity of TSP-1 or TSP-2 mRNA were noted in the contralateral (left) side or in sham-operated rats (data not shown). Similar results were duplicated in 2 other sets of animals.

**Figure 3.** Western blot demonstration of TSP-1 and TSP-2 expression at the protein level in the ischemic and contralateral cortex after focal cerebral ischemia/reperfusion. Transient ischemia for 60 minutes resulted in a time-dependent change in the protein level of TSP-1 and TSP-2 in the ischemic right cerebral cortex but not in the contralateral side. Sh denotes sham-operated controls; numeric values indicate duration of reperfusion (hours). The same blot was cut and hybridized with β-actin to serve as an internal control. A representative of 3 immunoblots with similar results is shown.
and incubated overnight at 4°C with either a monoclonal mouse anti-TSP-1 antibody (1:100, BD Transduction Laboratories) or a mouse monoclonal anti-TSP-2 antibody (1:100, BD Transduction Laboratories), followed by a biotin-labeled goat anti-mouse IgG (Vector Laboratories). After they were washed, the sections were incubated further with ABC Elite complex (Vector Laboratories). The staining was visualized with diaminobenzidine. Slides were washed, dehydrated, cleared in xylene, and mounted. Control slides were prepared in a similar manner, omitting the primary antibody.

**Double-Staining Immunohistochemistry**

Double-labeling methods to examine cellular origins of gene products have been reported elsewhere. Brain sections were permeabilized with 0.3% Triton X-100 and 4% normal goat serum in 0.01 mol/L PBS (pH 7.4) for 20 minutes. Monoclonal mouse anti-TSP-1 antibody (1:100, BD Transduction Laboratories) or mouse anti-TSP-2 antibody (1:100, BD Transduction Laboratories) was incubated with 1 of the polyclonal rabbit antibodies against von Willebrand factor (vWF; endothelial marker, 1:500, Sigma), platelet and endothelial cell adhesion molecule (PECAM-1; endothelial marker, 1:100, Boehringer-Mannheim), mitogen-activated protein (MAP)-2 (neuronal marker, 1:100, Santa Cruz), Mac-1 (macrophage marker, 1:100, Chemicon), or CPP32 (apoptosis marker, 1:100, PharMingen) and applied to the sections at 4°C overnight. The sections were then incubated with an FITC-conjugated goat anti-mouse IgG antibody (1:100, Vector Laboratories) and Texas Red–conjugated goat anti-rabbit antibody (1:100, Vector Laboratories). Slides were washed, wet-mounted, and examined under fluorescence microscopy.

**Statistical Analysis**

One-way ANOVAs followed by post hoc Fisher-protected t tests using GB-STAT 5.0.4 (Dynamic Microsystems) were used to compare the temporal expression of mRNAs. A value of P<0.05 was considered significant.

**Results**

The primer pairs used for PCR amplification of TSP-1 or TSP-2 each resulted in a single cDNA fragment when visualized with ethidium bromide on agarose gel. The cDNA fragments derived from PCR were eluted and subcloned into pCRII, which provides a 1-step cloning strategy for direct insertion of PCR products into plasmid vector (Invitrogen). Each amplified cDNA fragment was subjected to DNA sequencing analysis for its identity in GenBank. Results indicate a very high fidelity of PCR amplification. These cDNA fragments served as probes to detect TSP-1 and TSP-2 mRNA in subsequent studies.

The temporal expression profile of TSP-1 mRNA after ischemia/reperfusion was examined by Northern blot analysis (Figure 1). The RT-PCR–amplified probe detected a 6.0- and a 4.0-kb transcript in the ischemic right MCA cortex (Figure 1A). The intensity of the 4.0-kb transcript was stronger than that of the 6.0-kb species, especially in sham-operated rats (Figure 1A, lane 1, sh). Sixty-minute ischemia resulted in an early transient, but significant, increase in the expression of the 4.0-kb transcript (Figure 1A). Quantitative analysis indicates a 1.5-fold peak induction at 60 minutes after ischemia compared with the sham operation (Figure 1C). Probably because of a relatively low basal level, ischemia-induced increase in the expression of the 6.0-kb transcript was more robust. A biphasic expression of the 6.0-kb transcript was noted. Induction of this 6.0-kb transcript was observed as early as 30 minutes after ischemia, peaking at 60 to 90 minutes (a 6-fold increase compared with the sham operation), and then declined at 4 hours after ischemia. A second peak was noted between 24 and 36 hours after ischemia with about the same magnitude of increase (6-fold) as in the first peak (Figure 1A and 1C). No significant changes in expression intensity of either the 4.0- or 6.0-kb transcript were detected in the uninjured left MCA cortex.

The RT-PCR–amplified TSP-2 probe also detected 2 mRNA transcripts, 6.0 and 4.0 kb, in the right MCA cortex in the sham-operated rats (Figure 1D, lane 1, sh) and in the ischemic right MCA cortex (Figure 1D). Sixty-minute ischemia resulted in a delayed increase of both transcripts in the ischemic right MCA cortex, with a significant increase noted for both transcripts starting 1 week after ischemia. Quantitative analysis indicates 3.5- and 2.5-fold
increases for the 6.0- and 4.0-kb transcripts at their respective peaks compared with the sham-operated controls. No significant changes in expression intensity of either the 4.0- or 6.0-kb transcript were detected in the uninjured left MCA cortex.

In situ hybridization was conducted to further examine the regional expression of TSP-1 and TSP-2 mRNA (Figure 2). In this study, brain slices (25 μm) were obtained from sham-operated control rats or from rats subjected to 60-minute ischemia and 60-minute reperfusion, 1 or 7 days. TSP-1 mRNA signal intensities were very low in the brains of sham-operated control rats, except for the basal lateral amygdaloid nucleus, where the TSP-1 mRNA signal was very prominent. At 60 minutes after ischemia, a dramatic increase in signal intensity of TSP-1 mRNA was noted exclusively in the leptomeninges covering the ischemic right MCA cortex. At 1 day after ischemia, an increase in TSP-1 message was observed in several areas, including the ischemic MCA cortex (in particular, the margin demarcating the infarct), the peri-infarct cortical area, and the leptomeninges covering the ischemic MCA cortex. TSP-1 mRNA was also detected in areas near the amygdaloid nucleus, olfactory tract, and forebrain bundle of the contralateral hemisphere and in the indusium griseum, bilaterally. Basal levels of TSP-2 mRNA were also very low in sham-operated controls. At 7 days after ischemia, a significant increase in TSP-2 mRNA signal was observed in the ischemic right MCA cortex and the penumbral region. High TSP-2 mRNA signal intensity was also found in the leptomeninges covering the ischemic right MCA cortex and both lateral ventricles.

We also examined the expression of TSP-1 and TSP-2 at the protein level. The temporal expression profiles of TSP-1 and TSP-2 gene products were first studied with Western blot analysis (Figure 3). The specific antibodies used in the present study recognized a 190-kDa band TSP-1 and a 200-kDa band TSP-2 protein, respectively. Basal expression of TSP-1 protein was detected in both the right and left MCA cortices in sham-operated rats (Figure 3, lane Sh in the ischemic and contralateral side). The level of TSP-1 protein expression was transiently increased in the ischemic cortex 4 hours after ischemia and partially resolved at 24 hours after ischemia. A second wave of TSP-1 protein expression was noted at 72 hours after ischemia and then gradually returned to basal level at 168 hours after ischemia, corresponding to the biphasic expression of TSP-1 mRNA over time described above. No significant change in TSP-1 expression at the protein level was noted in the contralateral uninjured left MCA cortex. TSP-2 protein was barely detected in the MCA cortex of sham-operated rats (Figure 3, lane Sh in the ischemic and contralateral side). Sixty-minute ischemia resulted in a delayed appearance of TSP-2 protein in the ischemic right MCA cortex, starting 72 hours and peaking 336 hours after ischemia, corresponding to the monophasic expression of TSP-2 mRNA described above. No significant change in TSP-2 expression at the protein level was noted in the contralateral uninjured left MCA cortex.

The regional expression of TSP-1 and TSP-2 proteins was examined by immunohistochemistry. In the present study, coronal brain sections (25 μm) were obtained from rats subjected to 60 minutes of ischemia followed by 60 minutes, and 1, 3, and 7 days of reperfusion, as well as sham operation (sham, Figure 4). All the changes depicted were within the ischemic MCA cortex or corresponding region in the sham-operated controls. TSP-1 immunoreactivity was confined to the ischemic MCA cortex and, to a lesser extent, the adjacent regions, such as the external capsule. No TSP-1 immuno-
activity was noted in subcortical structures, such as the hippocampus. The expression of TSP-1 immunoreactivity evolved over time, appearing at 1 day, peaking at 3 days, and declining to basal level by 7 days. TSP-2 immunoreactivity was very low in the ischemic MCA cortex during early time points up to 1 day. Intense expression of TSP-1 immunoreactivity was noted at 3 days and especially at 7 days after ischemia.

To determine the cellular origins of TSP immunoreactivity, double immunostaining was conducted. All the changes depicted were within the ischemic region. At 60 minutes after ischemia, a marked increase in TSP-1 immunoreactivity was noted in the leptomeninges covering the ischemic right MCA cortex (Figure 5), where intense expression of TSP-1 mRNA was also noted (Figure 2) at this early time point. TSP-1 immunoreactivity was heavily colocalized with PECAM-1, a specific cellular marker for endothelial cells in this region. Colocalization of TSP-1/PECAM-1 was also occasionally noted in blood vessels in the ischemic MCA cortex (Figure 5).

At 3 days after ischemia, TSP-1 immunoreactivity only partially colocalized with vWF (another specific cellular marker for endothelial cells) in the penumbral region. Adjacent to blood vessels, TSP-1 immunoreactivity colocalized with glial fibrillary acidic protein (GFAP, a specific marker for astroglia). Occasional colocalization of TSP-1 immunoreactivity with MAP-2 (a specific marker for neurons) and Mac-1 (a specific marker for macrophages) was removed from the blood vessels in the ischemic MCA cortex (Figure 6). TSP-2 immunoreactivity also only partially colocalized with vWF, MAP-2, and Mac-1. Only very rare colocalization of TSP-2 and GFAP was noted in the same area (Figure 7).

TSP-1 is known to induce endothelial cell apoptosis.\textsuperscript{17,18,25} The expression of the active form of caspase-3 is a hallmark of apoptotic cell death. Double-labeling studies suggest colocalization of TSP-1 and caspase-3 immunoreactivity, primarily in endothelial cells along the vessel wall (Figure 8).

**Discussion**

It is known that ischemia induces angiogenesis, particularly in the penumbral region, and the extent of angiogenesis has been correlated with survival in stroke patients.\textsuperscript{1,2} The identification of key angiogenic molecules that positively regulate the vascular growth has led to the development of therapeutic angiogenesis by enhancing the expression of angiogenic factors in ischemic tissues.\textsuperscript{26,27} Angiogenic activity is a balance between angiogenic and angiostatic drives. The role...
of angiostatic factors in the regulation of postischemic angiogenesis has not been studied in the ischemic brain. In the present study, we report the initial finding that transient ischemia results in an early biphasic induction of TSP-1 and late induction of TSP-2. The RT-PCR–amplified TSP-1 probe recognized 2 TSP-1 mRNA transcripts, 4.0 and 6.0 kb. This is in line with a previous study reporting that 2 major species of TSP-1 mRNA were induced by macrophage colony-stimulating factor. In the present study, the expression of the 6.0-kb transcript is more robust than the 4.0-kb species in the ischemic cortex. This may be related to the notion that the basal level of the 6.0-kb transcript was lower than that of the 4.0-kb species. Ischemia led to a biphasic induction of the 6.0-kb transcript, which peaked at 1 to 1.5 and 24 to 72 hours after ischemia, respectively. Interestingly, in balloon vascular injury, Miano et al also showed similar biphasic expression of TSP-1 after aortic injury. The mechanism and physiological significance of this biphasic induction are not clear at present. Results from the in situ hybridization study showed that the TSP-1 mRNA expressed in the first peak was predominately localized in the leptomeninges covering the ischemic MCA cortex, whereas the TSP-1 mRNA expressed in the second peak was expressed mainly within the ischemic MCA cortex, penumbra, and the leptomeninges. Western blot and double immunostaining studies confirm TSP-1 expression at the protein level. Sixty minutes after ischemia, TSP-1 immunoreactivity was exclusively colocalized with PECAM-1–positive endothelial cells, whereas 3 days after ischemia, TSP-1 immunoreactivity was colocalized with vWF (endothelial)–, GFAP (glial)–, MAP-2 (neuronal)–, and Mac-1 (macrophage)–positive cells. The highest degree of colocalization was noted between TSP-1 and vWF or GFAP primarily near blood vessels. Interestingly, Cheung et al reported that GFAP expression also peaked 3 days after ischemia in the same region in this rat MCA occlusion model. Astrocytes regulate the maturation of the blood-brain barrier after reoxygenation. TSP expression has been also shown to increase within the glia limitans and surrounding axon fascicles in a temporal pattern along the path of axonal regeneration in goldfish optic nerves and therefore may be involved in central nervous system regeneration. Furthermore, astrocyte expression of TSP-1 contributed to the regulation of oligodendrocyte precursor migration. Thus, TSP-1 expression in astrocytes may be of significance in the remodeling of the ischemic brain. TSP-1 is produced and secreted by macrophage/microglia after nerve lesions, promoting neurite outgrowth and phagocytosis of degenerated neuronal material.
The expression of TSP-1 and TSP-2 appears inversely coupled to the progression of ischemia-induced angiogenesis. TSP-1 was upregulated during early reperfusion periods when low activity of angiogenesis was noted. The TSP-1 level was then reduced to basal levels, whereas angiogenesis reached its peak at 7 days after ischemia. TSP-2 expression occurred later and reached the plateau at 7 days after ischemia, when vascular density had reached the peak and was declining toward the preischemic level with the regression of newly formed large vessels. Our results suggest that the decline in TSP-1 expression to the basal level is coupled to massive angiogenesis and that the expression of TSP-2 is associated with the resolution of angiogenesis and progressive tissue liquefaction. Interestingly, TSP-1– and TSP-2–null mice had denser vascularity in skin wounds, and in TSP-2-null mice, the period of vascularization was prolonged, and the wounds healed more rapidly. On the other hand, transgenic overexpression of TSP-1, expected to retard angiogenesis, resulted in delayed healing. TSP-1 and TSP-2 worked synergistically to completely abolish carcinoma A431 cells to form tumors. Furthermore, it has been shown that TSP-1 can block fibroblast growth factor–induced and VEGF-induced angiogenesis. VEGF induced biphasic TSP-1 expression in the ischemic retina, probably conferring a negative-feedback mechanism in angiogenesis. Importantly, premature cessation of the VEGF stimulus led to regression of most acquired vessels. Whether blocking the induction of TSP-1 and/or TSP-2 will affect postischemic angiogenesis remains to be studied.

The angiostatic action of TSP is probably mediated by a mechanism involving receptor CD36-induced endothelial apoptosis by activating caspase-3. A double-labeling study showed colocalization of TSP-1 and caspase-3 immunoreactivity in blood vessels in the penumbral area 3 days after reperfusion. The colocalization of TSP-1 and caspase-3 to variable degrees in the vascular wall may reflect different paces of apoptosis among endothelial cells lining blood vessels in an evolving angiostatic process.

In summary, results from the present study demonstrate that focal cerebral ischemia/reperfusion leads to the expression of TSP-1 and TSP-2 at the mRNA and protein level. These 2 angiostatic factors exhibited different temporal and spatial expression profiles involving different cell types. The expression of TSP-1 occurred early in a biphasic fashion, in contrast to that of TSP-2, which occurred in a delayed monophasic manner. TSP-1 and TSP-2 expression appears to be inversely coupled to the evolution of angiogenesis. Together, these results suggested that TSP-1 and TSP-2 expression might contribute to the termination of postischemic angiogenesis.

**Acknowledgments**

This work was supported by grants from the National Science Council, Taiwan; Academia Sinica, Taiwan; and the National Institutes of Health (grants NS-28995, NS-32636, and NS-40525). We thank Brittany Herzog for her editorial assistance.
References


Differential Regulation of Thrombospondin-1 and Thrombospondin-2 After Focal Cerebral Ischemia/Reperfusion
Teng-nan Lin, Gyoeng-Moon Kim, Jean-Ju Chen, Wai-Mui Cheung, Yong Y. He and Chung Y. Hsu

Stroke. 2003;34:177-186
doi: 10.1161/01.STR.0000047100.84604.BA

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/34/1/177