Fibrin Contributes to Microvascular Obstructions and Parenchymal Changes During Early Focal Cerebral Ischemia and Reperfusion

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**Background and Purpose**
Ischemic cerebral injury is associated with activation of the blood coagulation cascade. To elucidate the contribution of fibrin formation to microvascular injury during focal cerebral ischemia and reperfusion, we have studied the time course and the localization of fibrin deposition in cerebral microvessels and the surrounding tissues during ischemia/reperfusion in a well-described nonhuman primate model.

**Methods**
Cerebral tissues from adolescent male baboons were examined after 2-hour middle cerebral artery occlusion (n=3) and after 3 hours of middle cerebral artery occlusion and 1-hour (n=6), 4-hour (n=3), and 24-hour (n=4) reperfusion; tissues from control primates (n=3) also were examined. Fibrin deposition was detected by immunohistochemical techniques using the fibrin-specific monoclonal antibody MH-1. The number and size distribution of microvessels associated with fibrin were quantified by video-imaging microscopy.

**Results**
Fibrin was associated with microvessels only in the ischemic zone where severe neuronal injury was documented, its frequency increasing with the reperfusion period (F_{4.24}=3.80, P<.05). Extravascular fibrin deposition was significantly increased by 24-hour reperfusion compared with the other subjects (P<.05). Preischemia infusion of the anti–tissue factor monoclonal antibody TF9-6B4 resulted in significant reduction of intramicrovascular fibrin (P<.038 versus no intervention) at 1-hour reperfusion but had no effect on extravascular fibrin deposition.

**Conclusions**
These results suggest that microvascular fibrin deposition accumulates in a time-dependent manner during focal cerebral ischemia/reperfusion and that exposure of plasma to perivascular tissue factor is partially responsible for occlusion formation. During ischemia the large plasma protein fibrinogen extravasates and interacts with parenchymal tissue factor, forming significant extravascular fibrin by 24 hours of reperfusion. (Stroke. 1994;25:1847-1854.)

**Key Words**
- cerebral ischemia
- reperfusion
- fibrin
- microvascular injury
- immunohistochemistry

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In the brain, experimental occlusion of the middle cerebral artery (MCA) is associated with a time-dependent series of events affecting both the microvasculature and the surrounding parenchyma. The intramicrovascular occlusive events that occur during reperfusion after global ischemia and after focal cerebral ischemia are of interest as they may relate to the nature and extent of the parenchymal injury. Recent work has suggested indirectly that, in addition to cellular elements, fibrin formation in situ may play a role in microvascular obstructions during focal ischemia and reperfusion. fibrin formation, which has received very little attention in this setting, may affect microvascular flow and may give clues to the generation of hemorrhage during ischemic injury.

Generation of fibrin from fibrinogen by thrombin occurs as plasma is exposed to activated platelets, vascular tissue factor (TF), or other stimuli. Thrombin may be formed when platelets are activated by platelet-activating factor or when endothelial injury exposes underlying collagen, or when polymorphonuclear (PMN) leukocytes become activated by any number of stimuli.

The possibility that fibrin may cause cerebral microvascular perfusion defects was suggested by the significant reduction in postreperfusion occlusions after preconditioning with heparin and ticlopidine in a nonhuman primate model of MCA occlusion/reperfusion. Those observations confirmed a similar effect on microvascular patency when heparin was used in a macaque model of 4-hour MCA occlusion/reperfusion. The potential contribution of TF activity to microvascular occlusion during focal cerebral ischemia/reperfusion was recently suggested by the observation that TF monoclonal antibody (MoAb) increased microvascular patency in the ischemia/reperfusion zone when infused before MCA occlusion. In a limited study using the same model, fibrin was found to be associated with PMN leukocytes and degranulated platelets in the obstructed capillaries.
Materials and Methods

Sixteen adult male baboons (Papio anubis/cynocephalus) weighing 8.0 to 11.4 kg were used for the MCA occlusion and occlusion/reperfusion studies, and 3 separate primates weighing 9.2 to 25.0 kg served as controls. Tissues from 15 of the animals (MCA occlusion, MCA occlusion/reperfusion, and controls) were from subjects reported previously.1 No animal had evidence of diseases at the time of surgery, nor were there abnormalities at histological examination. The specificity of the antibody to baboon fibrin was additionally confirmed by an enzyme-linked immunosorbent assay (ELISA) procedure, which identifies freely soluble fibrin. In this assay, soluble fibrin polymer is produced in vitro from citrated plasma by the addition of thrombin (0.025 NIH U/mL). MH-1 is used both as the capture and as the signal antibody conjugated to horseradish peroxidase for the colorimetric determination of soluble fibrin polymer (Fig 1).12 Previous studies using another detection system indicated a fourfold lower avidity of MH-1 to baboon fibrin than human fibrin.

Purification of the murine anti-human TF MoAb TF9-6B4 from cell culture for infusion has been described previously.5 The TF9-6B4 product (R.W. Johnson Pharmaceutical Research Institute, La Jolla, Calif) was more than 90% pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis and had endotoxin levels <2 endotoxin U/mL by standard limulus amebocyte lysate chromogenic assay (Whittaker).

Consecutive 10-μm cryostat sections from matched regions of the post-ischemia/reperfusion (right) and normal non-ischemia/reperfusion (left) basal ganglia separate from those previously reported7 were prepared for immunohistochemistry. Two separate blocks from each region per subject were chosen to provide at least six blocks per time point. Sections were fixed with methanol for 3 minutes at 4°C, immersed in 100 mmol/L glycine in phosphate-buffered saline (PBS; 100 mmol/L Na2HPO4 and NaH2PO4 and 140 mmol/L NaCl adjusted to pH 7.4) for 10 minutes, rinsed with PBS wash solution, and subsequently incubated with Blotto 20 to reduce nonspecific binding. For the MCA occlusion and MCA occlusion/reperfusion specimens, 50 μL of the primary antibody was incubated on each section for 120 minutes, washed, and then incubated with biotinylated horse anti-mouse immunoglobulin G (IgG; 1:400 in reagent diluent; Vector Laboratories) at 37°C for 30 minutes. Subsequent development of the peroxidase signal with the chromogen substrate 3-amino-9-ethyl carbazole (AEC Kit, Biomedica Corporation) was as described. Specimens from subjects that received the murine MoAb TF9-6B4 were stained separately using biotin-labeled purified MH-1 as the primary antibody to avoid the interaction of the horse anti-mouse IgG with TF9-6B4 in the tissues. Parallel experiments with selected specimens at 1-hour reperfusion using biotinylated MH-1 gave identical results with MH-1 as the primary antibody. All sections were counterstained with May-Grünwald–Giemsa.
Fig 2. Photomicrographs of localization of fibrin in peroxidase-stained sections or immunofluorescent preparations of the ischemic basal ganglia. Top left, Configuration 1: fibrin deposition limited to the intravascular space. Top right, Configuration 2: fibrin deposition in the intravascular lumen and the perivascular tissue. Bottom left and right, Configuration 3: fibrin network in the parenchymal tissues as in the ischemia/reperfusion zone. Microvessel-associated fibrin was confined to regions with severe neuron damage (98% type IV, see text). Magnification bar, 20 μm.

ner's hematoxylin (Biomedia Corporation) for 1 minute, blued in saturated sodium bicarbonate solution, and mounted. Controls routinely performed for each experiment included (1) deletion of the primary antibody, (2) deletion of the secondary antibody, and (3) the use of TIB115, a murine MoAb against the SV40 large T viral antibody, as an irrelevant primary antibody.

To further identify fibrin deposition in parenchymal tissues, adjacent sections were stained using the primary MoAb MH-1 with fluorescein isothiocyanate-conjugated anti-mouse IgG (Vector Laboratories).

The absolute number and minimum transverse diameters of manually identified microvessels containing CD31 antigen from the basal ganglia of post-ischemia/reperfusion and nonischemic territories were determined with the computerized video-imaging system described previously. Minimum transverse diameters of peroxidase-stained vascular structures from nonoverlapping images (×400) in a standard 1000-field matrix (66.4 mm²) were computed from each section with a linear measurement program. The number of vessels of diameter less than 100 μm stained by the MoAb CLB-HEC-75 was regarded as the total number of microvessels per field.1,17,21 Off-line analysis of the microvascular distribution was performed with resident statistical programs. Fibrin deposition is presented as the fraction of peroxidase-stained vessels with fibrin to those displaying CD31, and configuration 3 (fibrin lattices in the extravascular and/or parenchymal tissues only) (Fig 2). The presence of ischemic injury, indicated by the relative degree of neuron damage, was assessed by using a modification of the method of Eke et al22 as described previously. The 1000-field matrix was scanned for evidence of neuron damage, and this was correlated with fibrin deposition. Areas where type IV neuron silhouettes were found corresponded to variable degrees of visible tissue disturbance; tissue peripheral to those regions had some type II or III neuron damage.

All values are expressed as the mean or as the mean±SD. Data from at least six matched blocks per time point were analyzed. Statistical comparisons among the different time points were performed with ANOVA followed by Tukey's multiple comparison method. Data from the cohort that received TF9-6B4 was compared with the 1-hour reperfusion MCA occlusion/reperfusion data (no intervention) using Student's t test, one-tailed. Significance was set at P<.05 for all comparisons.

Results

Hemiparesis and/or unresponsiveness to tactile and visual stimuli contralateral to the MCA occlusion appeared within 5 to 10 minutes after MCA compression in each subject. A significant and persistent reduction in neurological score3 from 100 to 41.1±14.7 at 1 hour after MCA occlusion was observed in both MCA-occlusion and MCA-occlusion/reperfusion cohorts. Baseline blood cell count data were not different among cohorts or from those previously reported.
Table 1. Fraction of CD31-Positive Microvessels Associated With Fibrin (Fibrin/CD31)

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Duration, h</th>
<th>n</th>
<th>Control (−MoAb)</th>
<th>Fibrin</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Non-I/R</td>
<td>Post-I/R</td>
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<tr>
<td>Control</td>
<td>3</td>
<td>3</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
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<tr>
<td>MCA:O</td>
<td>2</td>
<td>3</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
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<tr>
<td>MCA:O/R</td>
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<td>3</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>MCA:O/R</td>
<td>4</td>
<td>3</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>MCA:O/R</td>
<td>24</td>
<td>4</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>+Anti-TF</td>
<td>1</td>
<td>3</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
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</table>

MoAb indicates monoclonal antibody; Non-I/R, nonischemic zone; Post-I/R, ischemia/reperfusion zone; MCA:O, middle cerebral artery occlusion only; MCA:O/R, 3-hour middle cerebral artery occlusion with indicated periods of reperfusion; Anti-TF, anti–tissue factor antibody; TF9-6B4, and Control (−MoAb), deletion of primary antibody (identical data were obtained with the irrelevant antibody TIB-115).

Values are mean±SD (per 1000 fields).

*Difference among the cohorts with post-I/R, F3,6=3.80, P<.05.
†P<.05 vs control, using Tukey’s multiple comparison method.

As noted in Fig 1, MH-1 cross-reacted with baboon fibrin with somewhat less avidity than human fibrin. In the ELISA, soluble baboon fibrin converted from fibrinogen by thrombin reacted approximately twofold less than soluble human fibrin with MH-1 but significantly more than control (P<.001).

In the control subjects, the vascular distribution of the endothelial epitope CD31 was not different in both basal ganglia and was the same as previously reported. No fibrin was detected in the intravascular or extravascular compartments of either basal ganglion (Table 1). CLB-HEC75 recognized 924.0±134.2 (n=6) microvessels in sections from both basal ganglia, which was consistent with the data from a previous study.

The vascular distributions of CD31 in both basal ganglia in each subject within the MCA-occlusion and MCA-occlusion/reperfusion cohorts were not different from those of the control subjects. Fibrin was not seen in the nonischemic basal ganglia of any MCA-occlusion or MCA-occlusion/reperfusion subject at any time point (Table 1). However, fibrin antigen was identified by MH-1 in the microvessels of the ischemic basal ganglia of all subjects after either MCA occlusion or MCA occlusion/reperfusion. The frequency of microvessels associated with fibrin relative to CD31 (fibrin/CD31) increased with ischemia and the duration of reperfusion: 4.8±3.7% at 2-hour MCA occlusion and 6.4±5.8% at 1-hour reperfusion, 10.1±10.8% at 4-hour reperfusion, and 14.3±6.6% at 24-hour reperfusion (Table 1, Fig 3). The mean frequency of microvessels with fibrin in configuration 1 ranged from 2.9% to 4.8% and was highest at 1-hour MCA occlusion/reperfusion. There was no significant difference in the mean frequency of microvessel-associated fibrin (4.5% to 8.3%) among the time points of reperfusion when configurations 1 and 2 were considered together. Parenchymal (extravascular) fibrin deposition in the ischemic basal ganglia increased successively after 1-hour reperfusion and was most prominent at 24-hour MCA occlusion/reperfusion (P<.05 for 24-hour MCA occlusion/reperfusion versus control, 2-hour MCA occlusion, and 1-hour and 4-hour MCA occlusion/reperfusion) (Table 2, Fig 3). Taken together the frequency of extravascular fibrin, whether in the perivascular space or the parenchyma, was also significantly increased at 24-hour MCA occlusion/reperfusion (configurations 2+3=11.0±5.2%) compared with that of the other subjects (P<.05) (Fig 3).

Fibrin was seen in microvessels of which 9.4% were 4.0 to 7.5 μm in diameter (capillaries), 86.2% were 7.5 to 30.0 μm in diameter, 4.0% were 30.0 to 50.0 μm in diameter, and 0.4% were 50.0 to 100.0 μm in diameter, indicating that fibrin was most commonly associated with microvessels in the range of 7.5- to 30.0-μm diameter. Microvessel-associated fibrin, whether intravascular, extravascular, or both, was confined to regions with type IV neuronal injury (98% type IV).

Infusion of the anti-TF MoAb TF9-6B4 just before MCA occlusion targeted perimicrovascular TF in the region of ischemic injury (Fig 4). TF9-6B4 significantly reduced fibrin deposition in the intravascular compartment (configuration 1) compared with the control co-
Table 2. Localization of Microvessel-Associated Fibrin (Fibrin/CD31)

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Duration, h</th>
<th>Non-I/R</th>
<th>Config 1</th>
<th>Config 2</th>
<th>Config 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>MCA:O</td>
<td>2</td>
<td>0.000±0.000</td>
<td>0.029±0.025</td>
<td>0.016±0.015</td>
<td>0.003±0.002</td>
</tr>
<tr>
<td>MCA:O/R</td>
<td>4</td>
<td>0.000±0.000</td>
<td>0.048±0.047</td>
<td>0.012±0.016</td>
<td>0.005±0.006</td>
</tr>
<tr>
<td>+ Anti-TF</td>
<td>24</td>
<td>0.000±0.000</td>
<td>0.033±0.020</td>
<td>0.050±0.030*</td>
<td>0.060±0.032†</td>
</tr>
<tr>
<td>Non-I/R</td>
<td></td>
<td>0.000±0.000</td>
<td>0.008±0.015t</td>
<td>0.009±0.018</td>
<td>0.003±0.005</td>
</tr>
</tbody>
</table>

Non-I/R indicates nonischemic zone; Post-I/R, ischemia/reperfusion zone; MCA:O, middle cerebral artery occlusion only; MCA:O/R, 3-hour middle cerebral artery occlusion with indicated periods of reperfusion; Anti-TF, anti-tissue factor antibody, TF9-6B4; and Config, configurations of fibrin deposition: 1, intravascular stain; 2, intravascular and extravascular stain; 3, extravascular stain.

*P<.05 vs control.
†P<.05 vs control, 2-hour MCA:O, 1-hour MCA:O/R, and 4-hour MCA:O/R.
$tP<.05$ vs control, 1-hour MCA:O/R.

The fate of fibrinogen in situ (through its conversion to fibrin) during focal cerebral ischemia and reperfusion has not been rigorously examined, although several experimental studies have suggested a role of thrombin activation in focal cerebral ischemia. This in situ fibrin localization study follows from an initial morphological survey of microvascular occlusion formation after MCA occlusion/reperfusion in the primate. (See also Fig 5 in del Zoppo et al.) Fibrin was only detected in microvessels of the ischemic basal ganglia where it accumulated in a time-dependent manner, being most prominent at 24 hours of reperfusion. The majority (approximately 86%) of microvessels containing fibrin were 7.5 to 30.0 μm in diameter, consistent with the known association of TF with microvessels greater than 10 μm in diameter in primate brain. It is evident from the anti-TF infusion experiments that perivascular TF is at least partially responsible for intravascular fibrin formation in this setting. This is supported by the additional finding here that increased permeability of microvessels during ischemia/reperfusion allowed binding of TF9-6B4 to perivascular TF (Fig 4). Unfortunately, because of the murine origin of both MH-1 and TF9-6B4, colocalization of fibrin and TF was not possible.

The generation of fibrin within the microvascular compartment during MCA occlusion and occlusion/reperfusion and the potential involvement of TF have important implications. For instance, intravascular fibrin formation may also be affected by the interaction of platelets with PMN leukocytes, by platelet activation, and/or by the activation of monocytes and vascular macrophages. P-selectin has recently been shown to mediate the interaction of granulocytes with platelets and with stimulated endothelium in the ischemic cerebral tissue and to separately promote granulocyte/endothelium-mediated fibrin deposition. The fraction of microvessels containing fibrin in this setting complements the previous findings of microvascular endothelial cell expression of P-selectin and intracellular adhesion molecule-1 (ICAM-1) in early post-ischemia/reperfusion. Thrombin promotes ICAM-1-dependent granulocyte adhesion to endothelial cells, suggesting that upregulation of ICAM-1 activity may also involve fibrin formation in and around the microvessels. A relation between endothelial cell P-selectin expression and fibrin formation, potentially through thrombin gen-
that fibrin deposition occurred as a secondary consequence of reduced regional cerebral blood flow and perivascular fibrin occlusion in cats. However, it cannot be excluded that fibrin deposition occurred as a secondary consequence of parenchymal damage. These experiments imply that extended reperfusion might therefore promote extravascular fibrin deposition. How the duration of ischemia might alter the ultimate fibrin deposition is not known. One issue raised in this model is the variation in fibrin deposition among separate subjects in the early reperfusion cohorts (Table 1). These variations were associated with differences in ischemic damage in those tissues. Thus, the effect of the anti-TF MoAb on extravascular fibrin deposition at longer reperfusion periods, as well as shorter ischemic periods, may be of interest.

In another example of experimental cerebral ischemia, fibrin deposits were recognized in the ischemic brain tissue of those animals who died of fatal cerebral ischemia and vasospasm.23 But in a rodent model of disseminated intravascular coagulation induced by continuous intravenous infusion of Escherichia coli endotoxin, fibrin deposition in cerebral vessels was uncommon.33 Fibrin deposits have also been seen in human acute renal failure of ischemic origin, perhaps a parallel situation.34-36 Fibrin deposition, mainly intravascular (Bowman's space) and tubular (urinary space), has been demonstrated in a rodent acute renal failure model after renal artery occlusion/reperfusion and could be prevented by heparin.48 Heparin or warfarin pretreatment significantly decreased renal fibrinogen and albumin content,37 and arwin improved glomerular filtration rate in separate studies with rat renal ischemia models.39

Although noncapillary microvessels were involved in these studies, it cannot be excluded that in some instances severe trauma to large vessels occurred by direct ischemic injury with consequent local leakage. This may offer one manifestation of ischemia-related hemorrhage. Among the various phenomena associated with microvascular perfusion disturbances in the brain after ischemia/reperfusion, fibrin deposition might be more sensitive to treatment than other causes of microvascular occlusion. In addition to treatment with anti-TF strategies, early use of antithrombins (eg, hirudin), platelet fibrinogen receptor (the α₅β₃-integrin, GPIIb/IIIa) antagonists, or fibrinolytic agents may alter fibrin formation in the cerebral microvasculature during or after focal cerebral ischemia and reperfusion. Determining whether and how such strategies may also affect extravascular fibrin formation should help to elucidate the origins of hemorrhagic transformation.

Acknowledgments

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References

Fibrin deposition after periods of cerebral ischemia may lead to microvascular obstructions and thus contribute to neuronal vulnerability. Okada and colleagues studied the time course and localisation of fibrin deposition after transient middle cerebral artery occlusion in baboons using a fibrin-specific monoclonal antibody. Fibrin formation increased with extended reperfusion periods and was restricted to damaged parenchymal areas. In addition, pretreatment with an anti-fibrin antibody reduced intravascular fibrin deposition.

This study is an extension of previous work from this laboratory on the microvascular consequences of transient focal ischemia. Previous investigations have documented the postischemic accumulation of polymorphonuclear leukocytes as well as the endothelial expression of P-selectin and intercellular adhesion molecule-1 (ICAM-1). Perivascular fibrin deposition implicates altered blood-brain barrier permeability in the pathogenesis of this injury model. Thus, it would be interesting to determine the spatial relations between leukocyte accum-
Fibrin contributes to microvascular obstructions and parenchymal changes during early focal cerebral ischemia and reperfusion.

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