Cerebral Microvascular Obstruction by Fibrin is Associated with Upregulation of PAI-1 Acutely after Onset of Focal Embolic Ischemia in Rats

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The mechanisms underlying cerebral microvascular perfusion deficit resulting from occlusion of the middle cerebral artery (MCA) require elucidation. We, therefore, tested the hypothesis that intravascular fibrin deposition *in situ* directly obstructs cerebral microcirculation and that local changes in type 1 plasminogen activator inhibitor (PAI-1) gene expression contribute to intravascular fibrin deposition after embolic MCA occlusion. Using laser-scanning confocal microscopy (LSCM) in combination with immunofluorescent staining, we simultaneously measured in three dimensions the distribution of microvascular plasma perfusion deficit and fibrinogen immunoreactivity in a rat model of focal cerebral embolic ischemia (n = 12). In addition, using *in situ* hybridization and immunostaining, we analyzed expression of PAI-1 in ischemic brain (n = 13). A significant (p < 0.05) reduction of cerebral microvascular plasma perfusion accompanied a significant (p < 0.05) increase of intravascular and extravascular fibrin deposition in the ischemic lesion. Microvascular plasma perfusion deficit and fibrin deposition expanded concomitantly from the subcortex to the cortex during 1 and 4 hr of embolic MCA occlusion. Three-dimensional analysis revealed that intravascular fibrin deposition directly blocks microvascular plasma perfusion. Vascular plugs contained erythrocytes, polymorphonuclear leukocytes, and platelets enmeshed in fibrin. *In situ* hybridization demonstrated induction of PAI-1 mRNA in vascular endothelial cells in the ischemic region at 1 hr of ischemia. PAI-1 mRNA significantly increased at 4 hr of ischemia. Immunohistochemical staining showed the same pattern of increased PAI-1 antigen in the endothelial cells. These data demonstrate, for the first time, that progressive intravascular fibrin deposition directly blocks cerebral microvascular plasma perfusion in the ischemic region during acute focal cerebral embolic ischemia, and upregulation of the PAI-1 gene in the ischemic lesion may foster fibrin deposition through suppression of fibrinolysis.

*Key words: stroke; plasminogen activator inhibitor; rat; fibrin; microvascular; perfusion; confocal microscopy*

Occlusion of the middle cerebral artery (MCA) results in progressive impairment of downstream cerebral microvascular plasma perfusion (Crowell and Olsson, 1972; Little et al., 1975; Buchweitz-Milton and Weiss, 1988; Ennis et al., 1990). Using intravascular fluorescent tracer molecules or fluorescent tracers in combination with laser-scanning confocal microscopy (LSCM), we and others have shown a significant reduction of cerebral microvascular plasma perfusion and concomitant cerebral injury in the ischemic core at 1 and 4 hr after MCA occlusion (Dawson et al., 1997; Zhang et al., 1999a). Data are emerging to suggest that intravascular fibrin deposition contributes to microvascular obstruction (Okada et al., 1994; Siebler et al., 1994; Heyes and Cervos-Navarro, 1996). For example, microvascular fibrin deposition accumulates during early focal cerebral ischemia, and reperfusion in the nonhuman primate and fibrin-containing microthromboemboli are found in acute human ischemic brain (Siebler et al., 1994; Heyes and Cervos-Navarro, 1996).

A fibrin thrombus is formed from fibrinogen by activation of thrombin (Collen and Lijnen, 1991; Loscalzo and Schafer, 1992). Endogenous fibrinolysis is mediated by plasminogen activators that convert the zymogen plasminogen into the active serine protease plasmin. Plasmin is the primary enzyme responsible for removal of fibrin deposits (Collen and Lijnen, 1991; Vassali et al., 1991; Plow et al., 1995). Type 1 plasminogen activator inhibitor (PAI-1) inhibits plasminogen activators *in vivo* (Loskutoff et al., 1989). PAI-1 is secreted by a variety of cells, including endothelial cells and platelets (Loskutoff et al., 1989; Braaten et al., 1993; Kollros et al., 1994; Stringer et al., 1994; Handt et al., 1996). Elevation of PAI-1 activity is associated with fibrin deposition after ischemia (Hamsten et al., 1987; Margaglione et al., 1994). Therefore, intravascular deposition of fibrin in ischemic brain suggests a perturbation of the procoagulant and fibrinolytic activity cascades.

Despite the increasing number of reports about the effects of fibrin deposition on microcirculatory impairment, information is lacking whether intravascular fibrin deposition *in situ* directly obstructs cerebral microcirculation and how local changes in PAI-1 gene expression contribute to intravascular fibrin deposition. In this report, we used three-dimensional LSCM in combination with immunofluorescent staining to investigate the effects of intravascular fibrin deposition on cerebral microvascular plasma perfusion deficits in a rat model of focal cerebral embolic ischemia (Zhang et al., 1997). In addition, using *in situ* hybrid-
Liz and immunostaining, we analyzed expression of PAI-1 in ischemic brain. Our data indicate that microvascular perfusion deficits after embolic stroke may be facilitated by increases in PAI-1 levels, leading to intravascular fibrin deposition.

MATERIALS AND METHODS

All experimental procedures have been approved by the Care of Experimental Animals Committee of Henry Ford Hospital. Animal model. Male Wistar rats (n = 25) weighing 300–350 gm were anesthetized with halothane (1–3.5% in a mixture of 70% N2O and 30% O2) given by face mask. The rectal temperature was maintained at 37°C throughout the surgical procedure using a feedback-regulated water heating system. The MCA was occluded by placement of an embolus at the origin of the MCA (Zhang et al., 1997). Briefly, a single intact fibrin-rich 24-h-old homologous clot (~1 μl) was placed at the origin of the MCA via a 15 mm length of modified PE-50 catheter. All ischemic animals were anesthetized after deparaffinizing, coronal sections (6 μm-thick) from paraffin-embedded tissue were incubated with the anti-fibrinogen antibody for 1 hr at room temperature. The sections were then incubated with biotinylated rabbit anti-goat IgG (Vector Laboratories). The immunoreactivity was visualized with diaminobenzidine.

In situ hybridization. The 562 bp mouse cDNA of PAI-1 was used as a probe for in situ hybridization (a gift from Dr. D. Belin, University of Geneva, Geneva, Switzerland) (Sappino et al., 1993). In situ hybridization was performed using a digoxigenin DNA labeling and detection kit (Boehringer Mannheim) according to manufacturer's protocol. Briefly, after deparaffinizing, coronal sections (6 μm) were digested by proteinase K (100 μg/ml) for 15 min at 37°C and were fixed by 10% formaldehyde for 5 min at 4°C. Prehybridization solution containing 4× SSC, 50% deionized formamide, 1× Denhardt's solution, 250 ng/ml of salmon sperm DNA, 10% dextran sulfate, and 0.25 mg/ml yeast tRNA was applied for 1 hr at room temperature. Denatured digoxigenin-labeled cRNA probe (2× SSC, 100°C) was hybridized with the sections at 50°C. After hybridization, the sections were incubated under coverslips overnight at 42°C with hybridization solution in a humidified chamber. Posthybridization stringency washes at room temperature included 2× SSC for 1 hr, 1× SSC for 1 hr, and 0.5× SSC for 1 hr. After treatment with 2% of normal sheep serum, the sections were incubated with biotinylated rabbit anti-digoxigenin antibody conjugated to alkaline phosphatase at a dilution of 1:500 in 2% normal sheep serum. The sections were then incubated with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution that was used as a color substrate. Color reaction time was 20 hr. Sections were dehydrated in a graded series of ethanol and mounted.

Three-dimensional image acquisition. The vibratome sections were analyzed with a Bio-Rad (Cambridge, MA) MRC 1024 (argon and krypton) laser-scanning confocal imaging system mounted onto a Zeiss microscope (Bio-Rad). With the FITC-perfused tissue samples from each rat, 10 vibratome sections from interaural 6.38 mm to interaural 1.00 mm (Paxinos and Watson, 1986) were used for immunohistochemical staining and for in situ hybridization.

Immunohistochemistry. A goat anti-mouse fibrinogen/fibrin antibody was used at a titer of 1:1000 to assess the deposition of fibrin and fibrinogen-related antigen in brain (Accurate Chemical & Scientific, Westbury, NY). Although this antibody detects both fibrin and fibrinogen, the titer of the antibody used in the present study primarily reacted with fibrin (Ploplis et al., 1995; Kitching et al., 1997). A rabbit anti-mouse PAI-1 antibody (a gift from D. J. Lockswoth, The Scripps Research Institute, La Jolla, CA) was used at a titer of 1:500 to assess the PAI-1 antigen (a gift from Dr. D. J. Lockswoth, The Scripps Research Institute, La Jolla, CA). A mouse monoclonal antibody to microtubule-associated protein-2 (MAP-2; clone AP20; Boehringer Mannheim, Indianapolis, IN) was used at a titer of 1:50 for evaluation of early ischemic neuronal injury (Dawson and Hallenbeck, 1996; Zhang et al., 1999a). A rabbit polyclonal antibody against cow globulin fibrillary acidic protein (GFAP) (1:400; Dako, Carpinteria, CA) was used for astrocytes. The immunospecificities of MAP-2 and GFAP antibodies have been well demonstrated in rats (Garcia et al., 1994; Dawson and Hallenbeck, 1996).

Single immunofluorescence labeling was performed to measure fibrin deposition. Vibratome sections were incubated with the anti-fibrinogen antibody for 3 d at 4°C, and sections were then incubated with the Cy3-conjugated anti-goat IgG antibody (Vector Laboratories, Burlingame, CA). Double immunofluorescence labeling for fibrin(ogen) and GFAP or fibrin(ogen) and MAP-2 was performed to simultaneously evaluate fibrin deposition and astrocytic reactivity or fibrin deposition and neuronal injury, respectively. Vibratome sections were incubated with the antibody against fibrinogen for 3 d at 4°C, and sections were then incubated with the secondary antibody conjugated to Cy5. These vibratome sections were incubated with the antibody against fibrinogen for 3 d at 4°C, and then with the secondary antibody conjugated to Cy3. Because vibratome coronal sections were perfused with FITC dextran, red Cy3 and far red Cy5 fluorochromes were used for immunofluorescence double-labeling. Control experiments consisted of staining brain coronal tissue sections as outlined above, but omitted the primary antibodies. Single immunofluorescence (GFAP or MAP-2)-stained sections were used to compare the staining patterns to those obtained in the double-stained sections. Although the titer of the antibody against fibrinogen used in the present study primarily reacted with fibrin, contaminating fibrinogen may be a problem in nonperfused tissue. To further confirm the specificity of this antibody for fibrin, immunostaining for fibrin(ogen) was performed on an additional set of rats that were extensively perfused as indicated above. Coronal sections from paraffin-embedded tissue (6 μm) were incubated with the anti-fibrinogen antibody for 1 hr at room temperature. The immunoreactivity was visualized with diaminobenzidine.

Three-dimensional image analysis and reconstruction. To quantify
FITC–dextran and fibrin(ogen) immunoreactivity in tissue samples, all FITC–dextran and fibrin(ogen)-immunoreactive images acquired from the LSCM were analyzed with The Microcomputer Imaging Device (Imaging Research, St. Catherines, Ontario, Canada) image analysis system, as previously reported (Morris et al., 1999; Zhang et al., 1999a). Briefly, a single composite three-dimensional image (260.6 × 260.6 × 20 μm³) was reconstructed from the distribution of FITC–dextran or fibrin(ogen) immunoreactivity. Because the z-step position was kept intact, the resulting reconstructions covered identical tissue volumes and could be overlaid to produce composite images. A fixed grayscale display cutoff of 60 for FITC–dextran or 150 for fibrin(ogen) immunoreactivity was then applied to the model to ensure that the three-dimensional reconstruction was an accurate rendering of the original tissue-staining pattern. The choice of a cutoff value of 60 for FITC–dextran was based on our previous studies (Morris et al., 1999; Zhang et al., 1999a). A cutoff value of 150 for fibrin(ogen) immunoreactivity was based on preliminary image analysis data. We used a series of cutoff values (20–200) for fibrin(ogen) immunoreactivity in our preliminary analysis and found that a cutoff value of 150 most faithfully reflects the original images. The total volume of staining present in the rendered cube of tissue was then calculated in cubic micrometers and divided by the total tissue volume to determine the percentage of tissue volume that was fluorescently marked.

To eliminate low-frequency variations in gray scale value in two dimensions and small size noise in three dimensions, all GFAP-immunoreactive images acquired from the LSCM were analyzed using Eigentool image analysis software on a SUN UltraSPARC2 workstation (SUNvision). Eigentool software, developed by the Image Analysis Laboratory at Henry Ford Hospital, has a comprehensive set of functions for analyzing images in two and three dimensions (Windham et al., 1988; Soltanian-Zadeh and Windham, 1994). After the volume was thresholded by a gray level of 120, three-dimensional objects were determined from the remaining voxels. A voxel was included in an object if a face, side, edge, or corner touched any voxel already in that object. The size of a three-dimensional object was the number of voxels contained in the object. All three-dimensional objects with fewer than two voxels were eliminated from further analysis (Zhang et al., 1999b). The measurement of GFAP immunostaining present in tissue was simply the number of voxels remaining in the volume. Data are presented as a percentage of volume, in which the number of GFAP-immunostained voxels was divided by the total number of voxels in the volume. These thresholds eliminate noise and do not alter the original signals (Zhang et al., 1999b). Binary images were generated from subimaging of GFAP-immunoreactive volume. These binary images were imported to the MCID image analysis system for constructing three-dimensional images.

Quantitation of PAI-1 mRNA and PAI-1-immunoreactive vessels. Each PAI-1-immunostained and PAI-1-hybridized coronal section was digitized under a 20 or 40× objective (BX40: Olympus Optical, Tokyo, Japan) for measurement of the number of PAI-1 mRNA and PAI-1-immunoreactive vessels and diameters of the vessels using a three-CCD color video camera (DVC-970MD; Sony, Tokyo, Japan) interfaced with an MCID image analysis system. Numbers of vessels exhibiting PAI-1 mRNA and PAI-1 immunoreactivity were counted throughout the brain, and the maximum diameter (the maximum internal distance perpendicular to the maximum curved chord) of these vessels was measured using the MCID system. Vessels were categorized by their diameters as: capillary (<7.5 μm), precapillary arterioles and postcapillary venules (7.6–30 μm), and small arterioles and connecting vessels (>31–50 μm) (del Zoppo, 1994).

Statistical analysis. ANOVA followed by t tests with Bonferroni correction were used to compare control, 1, and 4 hr groups. All data are presented as mean ± SE, and p < 0.05 was considered statistically significant.

RESULTS

Distribution of fibrin(ogen) immunoreactivity and cerebral microvascular plasma perfusion

To examine whether deposition of fibrin directly obstructs cerebral microvascular plasma perfusion, distribution of FITC–dextran-filled cerebral microvessels and Cy5-labeled fibrin(ogen) immunoreactivity was measured in three dimensions in the control and the embolic ischemic animals. FITC–dextran-filled microvessels in x–y projections exhibited an irregular and tortuous pattern in the sham-operated and nonsurgical brains (Fig. 1A, green). Intravascular blood cells were visible as dark oval figures filling the microvascular lumina between the intraluminal FITC–dextran (Fig. 1A). Fibrin(ogen) immunoreactivity was not detected in the control rat brains (Fig. 1B). In contrast, 1 hr after MCA occlusion, large areas of little or no FITC–dextran were primarily detected in the ipsilateral subcortex (Fig. 1D, green) and occasionally in the piriform cortex, suggesting nonperfused and underperfused tissue. An irregular tubular pattern of fibrin(ogen) immunoreactivity was observed in the areas with little or no FITC–dextran and extended to the areas with intraluminal FITC–dextran (Fig. 1E, red). Fibrin(ogen) immunoreactivity was not detected in the ischemic lesion when the primary antibody was omitted (Fig. 1G). Intraluminal FITC–dextran terminated abruptly within cerebral microvessels (Fig. 1D, green). Examination of this region under high-power magnification revealed intense fibrin(ogen) immunoreactivity (Fig. 1J–O, red) proximal to intraluminal FITC–dextran (Fig. 1J–O, green) in x–y, x–z, and y–z projections, indicating that intravascular deposition of fibrin locally blocked perfusion of FITC–dextran. In addition, three-dimensional reconstructions revealed that intravascular fibrin(ogen) immunoreactivity partially blocked intraluminal FITC–dextran perfusion upstream within relatively large vessels and led to complete obstruction of FITC–dextran perfusion downstream vessels (Fig. 2A,B). To further confirm the possible intravascular deposition of fibrin observed on three-dimensional images, fibrin(ogen) immunohistochemistry was performed on coronal sections from the extensively perfused brain tissue. Intravascular fibrin(ogen)-immunoreactive meshwork was also observed on veins (Fig. 2C,D) and capillaries (Fig. 2E) in extensively perfused brain tissue after 1 hr of MCA occlusion. Erythrocytes, polymorphonuclear (PMN) leukocytes, and platelets were attached to fibrin by multiple connections, and aggregated platelets were enmeshed in fibrin (Fig. 2C–E).

At 4 hr of MCA occlusion, the areas with little and no FITC–dextran in subcortex expanded to the cortex supplied by the MCA, and expansion of underperfused FITC–dextran areas was accompanied by an increase in fibrin(ogen) immunoreactivity. In addition to intravascular fibrin(ogen) immunoreactivity as seen at 1 hr of embolic stroke, three-dimensional reconstruction revealed massive irregular shapes of fibrin(ogen) immunoreactivity in the areas with no FITC–dextran in the subcortex (Fig. 2F, red), suggesting the presence of fibrin deposition in the parenchyma. To further confirm fibrin deposition, immunohistochemistry of fibrin(ogen) was performed on extensively perfused brain tissue. The fibrin(ogen)-immunoreactive meshwork in the subcortical parenchymal tissue colocalized with shrunken neurons and activated astrocytes in perfused brain tissue (Fig. 2G). This staining pattern was comparable to that seen in three-dimensional images, confirming deposition of fibrin in the parenchyma. The parenchymal fibrin(ogen) immunoreactivity was primarily detected in the subcortex. The intravascular fibrin(ogen) immunoreactivity was present in areas of the piriform and parietal cortex supplied by the MCA (Fig. 2H). These areas exhibited mixtures of non FITC–dextran, FITC–dextran perfusion, and fibrin(ogen) immunoreactivity (Fig. 2H).

To obtain quantitative data on levels of FITC–dextran and fibrin(ogen) immunoreactivity, we measured FITC–dextran and Cy5–fibrin(ogen) immunoreactivity in three-dimensional images obtained from LSCM. Values of FITC–dextran were 1.7 and 0.7% for the control cortex and subcortex (Fig. 2A,B), respectively, which are above previously published data (0.74–0.86% for...
The reason for these high values is that some images obtained from homologous tissue in the contralateral hemisphere contained large vessels, as indicated in Figure 2, A and B. If we exclude these large vessels, values of FITC–dextran were 1.1% for the cortex and 0.6% for the subcortex. A significant (\( p < 0.05 \)) reduction of FITC–dextran was accompanied by a significant increase in fibrinogen immunoreactivity in the ipsilateral subcortex at 1 and 4 hr and in the ipsilateral cortex at 4 hr after embolic stroke, compared with the homologous tissue in the contralateral hemisphere (Fig. 3A,B, respectively). Although a reduction in plasma perfusion and an increase in fibrinogen immunoreactivity were detected in the ipsilateral cortex, differences between the ipsilateral and the contralateral cortex were not statistically significant at 1 hr after MCA occlusion. However, fibrinogen immunoreactivity was significantly (\( p < 0.01 \)) higher in the ipsilateral cortex at 4 hr than fibrinogen immunoreactivity at 1 hr after MCA occlusion (Fig. 3A,B).
numbers of vessels that were fibrin(ogen)-immunoreactive under light microscopy (Table 1). A significant ($p < 0.05$) increase of intravascular fibrin(ogen) immunoreactivity was also detected in the cortex and the subcortex at 1 and 4 hr after stroke compared with the contralateral hemisphere. Numbers of intravascular fibrin(ogen)-immunoreactive vessels were significantly ($p < 0.05$) increased in the ipsilateral cortex at 4 hr after ischemia compared with the 1 hr group. Numbers of vessels with extravascular fibrin deposition were significantly ($p < 0.05$) higher in the subcortex at 4 hr after ischemia than at 1 hr and the control group (Table 1).

To examine neuronal response to plasma perfusion deficits and fibrin deposition, MAP-2 immunoreactivity was examined along with FITC–dextran and fibrin(ogen) immunoreactivity. Triple fluorescence in the $x$-$y$ projections revealed that intense fibrin(ogen) immunoreactivity (Fig. 4A, red) was present in areas of low MAP-2 immunoreactivity (Fig. 4A, blue) and little or no FITC–dextran (Fig. 4A, green) compared with the homologous tissue in the contralateral hemisphere (Fig. 4B). Analysis of extensively perfused brain tissue under light microscopy revealed that acute ischemic neuronal injury, dark neurons, were present adjacent to vessels with fibrin deposition at 1 hr after embolic stroke (Fig. 4C, arrowhead). At 4 hr after stroke, the subcortical areas with intravascular fibrin deposition exhibited increased numbers of shrunken neurons (Fig. 4D, arrowheads), swollen astrocytes (Fig.
Figure 3. Bar graphs show volumes of cerebral microvascular plasma (open bars) and fibrin(ogen) immunoreactivity (filled bars) in the cortex (A) and the subcortex (B) at 1 and 4 hr after embolic MCA occlusion. Control = homologous tissue in the contralateral hemisphere. *p < 0.05, significantly different from the control group; and +p < 0.01, significantly different from 1 hr group.

Table 1. Fibrin deposition in ischemic brain after embolic MCA occlusion

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cortex</th>
<th>Subcortex</th>
<th>Numbers of vessel with extravascular fibrin deposition</th>
<th>Numbers of vessels with intravascular fibrin deposition</th>
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<tbody>
<tr>
<td>1 hr (n = 6)</td>
<td>145 ± 45.9*</td>
<td>0</td>
<td>202 ± 121.67*</td>
<td>0</td>
</tr>
<tr>
<td>4 hr (n = 5)</td>
<td>408 ± 112.74*</td>
<td>0</td>
<td>417 ± 88.36*</td>
<td>0</td>
</tr>
<tr>
<td>control (n = 2)</td>
<td>0</td>
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All values are mean ± SE.

*Significantly (p < 0.05) different from the control group and from the contralateral hemisphere.

**Significantly (p < 0.05) different from the 1 hr embolic ischemic group.

Ipsi., Ipsilateral hemisphere; Contra., contralateral hemisphere.

Distribution of GFAP immunoreactivity
Activated astrocytes may contribute to microvascular impairment after focal cerebral ischemia (Hossmann, 1990). To examine whether activated astrocytes are involved in reduction of FITC-dextran perfusion, FITC-dextran, GFAP, and fibrin(ogen) immunoreactivity were simultaneously measured in three dimensions at 1 hr after MCA occlusion. GFAP immunoreactivity (Fig. 4I, blue) increased in areas with little or no FITC-dextran (Fig. 4I, green) and with increase of fibrin(ogen) immunoreactivity (Fig. 4I, red) when compared with the contralateral homologous areas (Fig. 4J). Although increased GFAP immunoreactivity surrounds microvessels with little FITC-dextran (Fig. 4I, blue), fibrin(ogen) immunoreactivity directly blocked FITC-dextran perfusion (Fig. 4K, red, arrows). Quantitative data (Fig. 5) show a significant (p < 0.05) reduction of FITC-dextran perfusion and significant increases in GFAP and fibrin(ogen) immunoreactivity in the subcortex compared with the homologous tissue in the contralateral hemisphere after 1 hr of MCA occlusion.

PAI-1 mRNA and PAI-1 immunoreactivity
PAI-1 mRNA was not detected in the brain tissue from sham-operated and nonoperated rats and in the contralateral hemisphere of ischemic rats examined by in situ hybridization (Fig. 6D). A hybridization signal for PAI-1 mRNA was detected in cells that had the appearance of endothelial cells and lined the surfaces of the vascular lumina in the ischemic lesion (Fig. 6A, arrows). Dense PAI-1 immunoreactivity appeared in the endothelial cytoplasm on venules (Fig. 6B, arrows) and capillaries (Fig. 6C, arrow) in the ischemic areas. To obtain quantitative data on levels of PAI-1 mRNA and PAI-1 antigen, we measured number of vessels and diameters of vessels that contained PAI-1 mRNA or PAI-1 immunoreactivity. Numbers of vessels that expressed PAI-1 mRNA were 4 ± 0.5 and 20 ± 12 in the ipsilateral subcortex at 1 and 4 hr of embolic MCA occlusion, respectively, and 0 ± 0 and 8 ± 2.6 in the cortex at 1 and 4 hr of ischemia, respectively. Numbers of vessels with PAI-1 immunoreactivity significantly increased in the ipsilateral subcortex (45 ± 9.0) and cortex (23 ± 7.9) at 4 hr when compared with number of vessels in the ipsilateral subcortex (3 ± 0.5) and cortex (0 ± 0) at 1 hr of embolic MCA occlusion. Seventy percent of PAI-1-immunoreactive cerebral vessels had a mean diameter of 19.1 ± 3.02 μm, 29% had a mean diameter of 5.2 ± 0.8 μm, and 1% had a mean diameter of 32 ± 2.8 μm, suggesting increases in PAI-1 primarily localize in precapillary arterioles, postcapillary venules, and capillaries (del Zoppo, 1994).
DISCUSSION
To directly address whether fibrin deposition obstructs cerebral microvascular plasma perfusion, we simultaneously measured in three dimensions microvascular plasma perfusion and fibrin deposition in ischemic brain at 1 and 4 hr of embolic MCA occlusion using intravascular fluorescent tracer molecules and immunofluorescent staining in combination with LSCM. The time points of 1 and 4 hr chosen in the present study were based on our previous studies in this model that a progressive cerebral microcirculatory impairment is present during this period (Zhang et al., 1997, 1999a; Morris et al., 1999). Three-dimensional reconstructions demonstrate that fibrin deposition not only directly obstructed microvascular plasma perfusion, primarily within capillaries of the ischemic core, but that fibrin was also present within plasma-perfused vessels bordering the ischemic core at 1 hr after MCA occlusion. The marked increase of intravascular fibrin deposition in the cortex and appearance of extravascular fibrin deposition in the striatum were associated with reduction of cerebral microvascular plasma perfusion at 4 hr of ischemia. This three-dimensional assessment was confirmed by measuring numbers of fibrin(ogen)-immunoreactive vessels on immunohistochemically stained sections. Numbers of vessels with intravascular fibrin deposition in the cortex and parenchymal fibrin deposition in the striatum are significantly higher at 4 hr of MCA occlusion than at 1 hr of ischemia. These data suggest that cerebral microvascular plasma perfusion secondary to MCA occlusion is an ongoing process that expands from the subcortex to the cortex over time of ischemia, and intravascular fibrin deposition directly causes this progressive cerebral microcirculatory impairment. Our finding of a progressive microvascular plasma perfusion deficit after ischemia is consistent with previous studies of embolic ischemia in mice and of permanent MCA occlusion in rats (Dawson et al.,

![Figure 4. Fibrin deposition and ischemic cell damage.](image_url)

**Figure 4.** Fibrin deposition and ischemic cell damage. *A* and *B* are images (x-y projections, 260.6 × 260.6 μm²) through the stack of 20 optical sections (1 μm/section) of plasma perfusion in capillary networks (green), fibrin(ogen) immunoreactivity (red), and MAP-2 immunoreactivity (blue) from a rat subjected to 1 hr of MCA occlusion. Increase in fibrin(ogen) immunoreactivity (*A*, red) and loss of plasma perfusion (*A*, green) and MAP-2 immunoreactivity (*A*, blue) on the ipsilateral hemisphere are evident (*A*) compared with the contralateral hemisphere (*B*). Dark neurons (*C*, arrowhead), shrunken neurons (*D*, arrowheads), and swollen astrocytes (*D*, arrow) were present in the striatum with intravascular fibrin deposition from extensively perfused brains at 1 (*C*) and 4 (*D*) hr of embolic MCA occlusion. Shrunken neurons (*arrowhead*) with vacuoles were present in the striatum with extravascular fibrin deposition (*E*) compared with intact neurons (*arrowhead*) in the contralateral striatum with patent vessels (*curved arrow*) at 4 hr of embolic MCA occlusion (*F*). Shrunken neurons (*arrowheads*), intact neurons (*curved arrow*), and swollen astrocytes (*arrow* were present in the cortex with intravascular fibrin deposition (*G*) compared with intact neurons (*arrowhead*) in the contralateral cortex with patent vessels (*curved arrow*) at 4 hr of ischemia (*H*). *I*-L are three-dimensional reconstructions of microvascular plasma perfusion (green), fibrin(ogen) immunoreactivity (red), and GFAP immunoreactivity (blue) in the caudate putamen from a rat subjected to 1 hr of embolic stroke. Enlargement of GFAP-immunoreactive cell bodies and processes (blue) surrounded vessels (green) in the ischemic region (*I*) compared with the homologous tissue in the contralateral hemisphere (*J*). Microvascular plasma perfusion (*K*, green, arrows) was directly blocked by fibrin deposition (*K*, red, arrows) when GFAP immunoreactivity was removed (*K*, *L*). The image size is 260.1 × 260.1 × 20 μm³ for *I*-K. Scale bar: *C*-*H*, 10 μm.
In addition, our data demonstrate that intravascular fibrin deposition is a primary cause for microvascular plasma perfusion deficit. The potential contribution of fibrin deposition to microcirculatory obstruction was suggested, but not proven, by the observation of fibrin deposition in cerebral microvessels and the parenchyma of the ischemic core in a nonhuman primate model of transient MCA occlusion (Okada et al., 1994). Transcranial Doppler studies reveal a high frequency of asymptomatic microemboli in stroke patients (Siebler et al., 1994). A recent histopathological study confirmed the presence of large numbers of fibrin containing microthromboemboli in the tissue bordering the necrotic brain for stroke patients who died within 7 d of ictus (Heye and Cervos-Navarro, 1996).

Our data show that microvascular plasma perfusion deficit and intravascular fibrin deposition was primarily colocalized in the subcortex with acute ischemic neuronal damage and activated astrocytes at 1 hr after embolic MCA occlusion. Microvascular plasma perfusion deficit and intravascular fibrin deposition expanded from the subcortex, with severe ischemic cell damage, to the cortex, with less ischemic cell damage, at 4 hr of MCA occlusion, suggesting that acute cerebral microvascular plasma perfusion deficit caused by fibrin deposition may contribute to ischemic cell damage. Our data are in agreement with other experimental findings (Pappata et al., 1993; Heiss et al., 1994; Garcia et al., 1995). CBF values immediately after an MCA occlusion are most reduced in the striatum, and this reduction of CBF expands to the cortex until several hours after MCA occlusion (Pappata et al., 1993; Heiss et al., 1994). There is a significant difference between the percentage of necrotic neurons identified in the striatum (>90%) compared with the cortex (40%) at 24 hr after MCA occlusion (Garcia et al., 1995). Moreover, the possibility that early fibrin deposition may contribute to the evolution of the ischemic lesion has clinical implications (Sherman, 1999).

Activated astrocytes appear to constrict large vessels in the ischemic region after 2 hr of embolic ischemia (Zhang et al., 1999b). In the present study, double immunofluorescent staining

Figure 5. Bar graph shows volumes of perfused cerebral microvascular plasma (open bars), fibrin(ogen) immunoreactivity (hatched bar), and GFAP immunoreactivity (filled bars) at 1 hr of embolic MCA occlusion. CS, Contralateral striatum; IS, ipsilateral striatum.

Figure 6. Endothelial cells express PAI-1. PAI-1 mRNA (A, arrows) and PAI-1 antigen (B, C, arrows) were present in the cytoplasm of endothelial cells in venules (A, B) and capillaries (C) in the ipsilateral striatum compared with PAI-1-immunonegative vessels in the contralateral hemisphere (D, arrow) at 4 hr of embolic MCA occlusion. Immunoreactivity of PAI-1 was visualized by diaminobenzidine.
for fibrinogen and GFAP revealed that astrocytes are activated at 1 hr of ischemia, as measured by a significant increase of GFAP immunoreactivity. Activated astrocytes surround microvessels that are fibrin-immunoreactive, suggesting that reactive astrocytes may also contribute to cerebral microcirculatory impairments during the early stage of embolic stroke by constricting cerebral vessels.

The specificity of the anti-fibrinogen antibody that we used for fibrin detection is consistent with previous reports on this antibody in a mouse model of thrombosis (Farrehi et al., 1998). In the present study, we found that fibrinogen immunoreactivity was only detected in the ischemic region in both nonperfused and present study, we found that fibrinogen immunoreactivity was present in the ischemic region in both nonperfused and extravascular parenchymal tissue in the ischemic region. Fibrin(ogen)-immunoreactive meshwork was found within vessels only detected in the ischemic region in both nonperfused and

Our results are consistent with the recent finding that PAI-1 gene in the endothelial cells may foster fibrin deposition through suppression of fibrinolysis. These data suggest that local perturbation of procoagulant and fibrinolytic genes in the brain may be important for cerebral microcirculatory impairment during early focal embolic cerebral ischemia.

REFERENCES


