Downstream Microvascular Thrombosis in Cortical Venules Is an Early Response to Proximal Cerebral Arterial Occlusion

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Background—Previous experimental studies have shown that downstream microvascular thromboinflammation is involved in brain damage from acute ischemic stroke. Using intravital microscopy, we investigated and characterized the sequence of downstream microvascular thromboinflammation in an ischemia/reperfusion acute ischemic stroke model.

Methods and Results—Rats underwent transient monofilament middle cerebral artery (MCA) occlusion. Cerebral microcirculation in the MCA territory was exposed through a craniotomy and analyzed using real-time intravital imaging coupled with laser Doppler interferometry. Leukocytes, platelets, fibrinogen, and blood–brain barrier permeability were analyzed by intravenous injection of fluorescent antibodies and bovine serum albumin. MCA occlusion induced a sudden and profound drop in downstream microvascular blood flow associated with leukocyte margination in the venous compartment. Leukocyte margination fostered fibrinogen deposition and thrombosis in postcapillary venules. Either in venules or arterioles, blood flow was not fully restored after MCA recanalization. Furthermore, venular thrombi persisted despite MCA recanalization, and leukocyte extravasation continued to develop in venules in association with blood–brain barrier disruption. Finally, microhemorrhages were occasionally observed, colocalizing with thrombosed venules characterized by marked leukocyte margination.

Conclusions—We showed that microvascular thrombosis in transient monofilament MCA occlusion and blood–brain barrier disruption are initiated immediately after occlusion and are propagated through the venous compartment in close association with marginating leukocytes. MCA occlusion–induced downstream microvascular thromboinflammation response was responsible for incomplete reperfusion after MCA recanalization and delayed microhemorrhages. (J Am Heart Assoc. 2018;7:e007804. DOI: 10.1161/JAHA.117.007804.)

Key Words: blood–brain barrier • leukocyte • middle cerebral artery occlusion • no-reflow • venous thrombosis

E xperimental studies using animal models of cerebral ischemia/reperfusion have provided broad evidence that proximal arterial occlusion causes deleterious secondary events that significantly contribute to acute ischemic stroke (AIS) pathophysiology. In particular, cerebral ischemia/reperfusion is known to trigger interactions among the vessel wall, platelets, leukocytes, and coagulation that can impair reperfusion and destabilize the blood–brain barrier (BBB). How and when these interactions, grouped under the term thromboinflammation, take place remains unclear. At present, the dominant paradigm is that they would mostly develop during the reperfusion phase and contribute to reperfusion injury. We observed in a recent study that leukocyte accumulation, mainly neutrophils, in microvessels of the ischemic hemisphere occurred as early as 30 minutes after proximal artery occlusion. The latter phenomenon was also reported in earlier studies including models of permanent focal cerebral ischemia, indicating that the thromboinflammatory response to cerebral ischemia was not necessarily the consequence of reperfusion. Limiting thromboinflammation has been proposed as a possible strategy to improve penumbra salvage in AIS. A better understanding of AIS-associated thromboinflammation could thus help in designing novel treatment for AIS. In this study, using intravital microscopy coupled with laser Doppler interferometry, we investigated and characterized the sequence of downstream microvascular responses to cerebral ischemia/reperfusion

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An accompanying Video S1 is available at http://jaha.ahajournals.org/content/7/5/e007804/DC1/inline-supplementary-material-1.avi

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Distal branches of the middle cerebral artery (MCA) of rats
Real-Time Intravital Imaging Coupled With Laser
occlusion (MCAO), as described previously.7 All experimental
120 minutes of transient mono
Male Sprague-Dawley rats (Janvier, France) underwent
Reperfusion
Middle Cerebral Artery Occlusion and
Reperfusion
Male Sprague-Dawley rats (Janvier, France) underwent
120 minutes of transient monofilament middle cerebral artery
occlusion (MCAO), as described previously.7 All experimental
procedures were declared to the French ministry of research
and authorized after ethics review (no. 20160125164052;
APAFIS 3792).

Real-Time Intravital Imaging Coupled With Laser
Doppler Interferometry
Distal branches of the middle cerebral artery (MCA) of rats
were exposed through a 4×4-mm craniotomy performed in
the right temporoparietal cortex with a hand-held drill, as
described previously.7 Cerebral microcirculation was directly
visualized using a fluorescence macroscope (MacroFluo; Leica
Microsystems) equipped with a heating plate with a ther-
omstat and a ×5 objective and connected to a scientific
CMOS camera (ORCA-Flash4.0; Hamamatsu Photonics). Data
acquisition and analysis were done using Metamorph software
(Molecular Devices).

All fluorescent markers were administered intravenously
into the tail vein. Rhodamine 6G was used to label
leukocytes and platelets, FITC (fluorescein isothiocyanate)–
conjugated polyclonal rabbit anti–human fibrinogen was used to
stain fibrinogen, Alexa 555–conjugated hamster anti–rat
CD42d was used to stain platelets, and Alexa Fluor 647–
conjugated BSA was used to assess vascular permeability.
Fluorescent BSA was injected intravenously 5 minutes before
recanalization.

Cortical vessel diameter before MCA occlusion and 1 hour
after recanalization was measured in intravital video micro-
scopy images using ZEN software (Zeiss).

Blood cell velocity in microvessels was measured using a
single-point laser Doppler vibrometer with an integrated CCD
video camera (CLV-2534; Polytec) and mounted on the
macroscope to monitor laser spot positioning. To allow
specific measurement of blood cell velocity, breathing-related
and flow-induced vessel wall vibrations were identified by
their bidirectional associated signal and eliminated by apply-
ing a low-pass filter set at 2 kHz. Frequencies due to the
unidirectional out-of-plane vibrations caused by circulating
blood cells were converted to speed according to the formula
v=(Δf×λ)/(2×cos(α)), where v is the blood cell velocity, Δf is
the Doppler-frequency shift, λ is the wavelength of the
 emitted wave (633 nm), and α is the angle between the blood
cell direction and the incident laser beam, which was
estimated at 80° (Figure 1). Data were recorded and treated
using the Polytec Vibrometer Software.

Statistical Analysis
Red blood cell velocities were compared using the nonpara-
metric Wilcoxon signed rank test for paired samples. Values
of P<0.05 were considered statistically significant.

Results
MCAO Induces Sudden and Profound Blood Flow
Anomalies Associated With Marked Leukocyte
Margination in Downstream Microvessels
We first analyzed the impact of middle cerebral artery
occlusion (MCAO) on blood flow in downstream pial microves-
sels. MCAO caused a sudden and profound drop in venous

Materials and Methods

The data and analytic methods and the study materials will be
made available to other researchers on request for purposes
of reproducing the results or replicating the procedure.

Clinical Perspective

What Is New?

• Downstream thromboinflammation is an early event trig-
  gered immediately after occlusion of a large proximal artery.
• It is characterized by leukocyte margination in cortical
  microvessels where blood flow is markedly reduced despite
collateral circulation from pial anastomoses.
• Leukocyte margination fosters fibrinogen deposition and
  secondary venous thrombosis, as well as rupture of the
  blood–brain barrier in both cortical venules and arterioles.
• Thrombi persisted and leukocyte extravasation continued
  progressing in venules after recanalization, which only
  partially corrected blood flow in cortical microvessels.
• In arterioles, despite recanalization-induced leukocyte
  washout, vascular leakage developed at sites where
  neutrophils had accumulated during the occlusion period.

What Are the Clinical Implications?

• Our results underscore the possible difficulty of reversing
  the microvascular consequences of large vessel occlusion
  because they are only partially corrected by proximal
  recanalization.
• The results further suggest that early actors of the
  thromboinflammatory cascade should be targeted as soon
  as possible and before proximal recanalization for maximal
  efficacy in the treatment of acute ischemic stroke caused by
  large vessel occlusion.

with particular respect to blood flow, microvascular thrombo-
sis, and BBB damage.
blood flow that was concomitant with a marked leukocyte margination (Figure 2A and 2B) that continued to develop throughout the occlusion period (Figure 2A). In downstream arterioles, MCAO led to a drop in blood flow (Figure 2A and 2C) that was also accompanied by leukocyte adhesion, albeit to a much lesser extent than in venules (Figure 2A). Remarkably, during MCAO, momentary and recurrent inversions of blood flow direction were systematically observed in downstream arterioles because of competitive flow coming from pial anastomoses (Figure 2A, Video S1).

MCA recanalization instantaneously restored downstream blood flow in both venules and arterioles, which corrected blood flow direction anomalies in arterioles (Figure 2A, Video S1). Nevertheless, blood flow was not fully restored by recanalization in either venules or arterioles, as red blood cell velocities after MCAO only reached \( \approx 70\% \) of their baseline.
Leukocyte Margination Fosters Downstream Thrombosis and BBB Disruption

The fact that blood flow in downstream microvessels was only partially restored by MCA recanalization indicated that secondary responses to MCAO likely impaired full reperfusion. We thus investigated signs of secondary thrombosis in downstream microvessels. Fibrinogen deposits were consistently observed on marginating leukocytes in postcapillary venules (Figure 3A). Fibrinogen deposition onto leukocytes was initiated during the MCAO period and eventually led to the formation of occlusive thrombi in postcapillary venules (Figure 3B). Of note, these MCAO-induced secondary thrombi persisted despite MCA recanalization (Figure 3B).

In addition to foster intravascular fibrinogen accumulation, sites of leukocyte adhesion and/or extravasation in venules and arterioles were also associated with BBB disruption, as indicated by the leakage of intravenously injected fluorescent BSA (Figure 3C). Whereas increased permeability to BSA from downstream venules and arterioles occurred systematically, microhemorrhages were much more occasional. Microhemorrhage was observed in 2 of 10 rats and developed from occluded postcapillary venules (Figure 3D).

Discussion

In this study, we showed that leukocyte margination is an early event that is triggered by arterial occlusion. Leukocyte margination starts concomitantly with the abrupt and profound drop in downstream arterial and venous blood flow that occurs despite collateral circulation provided by pial anastomoses. MCAO-induced leukocyte adhesion and extravasation mostly affects postcapillary venules and drives secondary venous thrombosis by promoting the formation of intravascular fibrinogen deposits onto the surface of adherent leukocytes. The latter observation supports previous findings showing that neutrophil depletion improved microvascular perfusion following MCAO.8

In agreement with an earlier study by del Zoppo and Mabuchi,1 we showed recently that neutrophils represented...
the vast majority of leukocytes being recruited to the ischemic hemisphere microvasculature during AIS.4,7 A previous study using intravital microscopy to investigate leukocyte–endothelium interactions also reported that neutrophil recruitment to the ischemic brain was predominant in the venous compartment.6 The thrombosis pattern we described in this study, with a phenomenon of venous thrombosis developing in a context of stasis and neutrophil margination, strongly recalls that observed in animal models of deep vein thrombosis.9 Strengthening the resemblance with deep vein thrombosis, in which VWF (von Willebrand factor) and neutrophil extracellular traps have been shown to play an important role, recent studies have shown that both VWF and thrombin stimulate neutrophil extracellular traps to form in vitro.10 Importantly, MCA recanalization only partially corrected MCAO-induced microvascular anomalies. In fact, at 1 hour after recanalization, red blood cell velocities in cortical arterioles and venules remained below baseline values. Although compensatory vasodilation occurred in both types of vessel, its limited magnitude was not sufficient to restore normal blood flow following transient monofilament MCAO. Moreover, venous thrombosis persisted and leukocyte extravasation continued progressing in venules after recanalization. In arterioles, despite the almost complete washout of adhering leukocytes following recanalization, vascular leakage developed at sites where neutrophils had accumulated during the occlusion period. These results underscore a possible difficulty in reversing MCAO-triggered downstream microvascular responses.

Our results also suggest that the deleterious role played by neutrophils at the acute phase of ischemic stroke might not be limited to thrombosis propagation and may expand to BBB disruption and hemorrhagic transformation. In fact, we showed in this study that BBB disruption originates from sites of neutrophil recruitment. As reported previously, hemorrhagic transformation in the form of petechiae is promoted by hyperglycemia, which primes neutrophils for activation and interactions with endothelial cells.11 In the same study, hemorrhagic transformation originated from areas showing persistent hypoperfusion in magnetic resonance imaging despite recanalization. Taken together, these observations suggest that hemorrhagic transformation might be a consequence of persisting occlusion rather than reperfusion. Supporting this hypothesis, using intravital microscopy in the present study, we observed microhemorrhages developing from occluded postcapillary venules.

In conclusion, we showed that microvascular thrombosis and BBB disruption in transient monofilament MCAO are initiated immediately after occlusion and preferentially develop in the venous compartment in close association with leukocyte margination.

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Disclosures
None.

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