Leukocyte Accumulation and Hemodynamic Changes in the Cerebral Microcirculation During Early Reperfusion After Stroke

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Background and Purpose—Leukocytes contribute to cerebral ischemia-reperfusion injury. However, few experimental models examine both in vivo behavior of leukocytes and microvascular rheology after stroke. The purpose of the present study was to characterize patterns of leukocyte accumulation in the cerebral microcirculation and to examine the relationship between leukocyte accumulation and microcirculatory hemodynamics after middle cerebral artery occlusion and reperfusion (MCAO-R).

Methods—Male rats (250 to 350 g) were anesthetized and ventilated. Tail catheters were inserted for measurement of arterial blood gases and administration of drugs. Body temperature was maintained at 37°C. Animals were subjected to 2 hours of MCAO by the filament method. A cranial-window preparation was performed, and the brain was superfused with warm, aerated artificial cerebrospinal fluid. Reperfusion was initiated by withdrawing the filament, and the pial microcirculation was observed by use of intravital fluorescence microscopy. Leukocyte accumulation in venules, arterioles, and capillaries; leukocyte rolling in venules; and leukocyte venular shear rate were assessed during 1 hour of reperfusion.

Results—We found significant leukocyte adhesion in cerebral venules during 1 hour of reperfusion after 2 hours of MCAO. Leukocyte trapping in capillaries and adhesion to arterioles after MCAO-R tended to increase compared with controls, but the increase was not significant. We also found that shear rate was significantly reduced in venules during early reperfusion after MCAO.

Conclusions—A model using the filament method of stroke and fluorescence microscopy was used to examine white-cell behavior and hemodynamics in the cerebral microcirculation after MCAO-R. We observed a significant increase in leukocyte rolling and adhesion in venules and a significant decrease in blood shear rate in the microcirculation of the brain during early reperfusion. Leukocytes may activate and damage the blood vessels and surrounding brain cells, which contributes to an exaggerated inflammatory component to reperfusion. The model described can be used to examine precisely blood cell–endothelium interactions and hemodynamic changes in the microcirculation during postischemic reperfusion. Information from these and similar experiments may contribute to our understanding of the early inflammatory response in the brain during reperfusion after stroke. (Stroke. 2000;31:1153-1161.)

Key Words: blood flow velocity □ leukocytes □ microscopy, fluorescence □ middle cerebral artery occlusion □ reperfusion □ rats

Stroke is the leading cause of adult disability and remains the third leading cause of death in the United States.1 Fortunately, more patients are experiencing reperfusion after stroke because of the availability of thrombolytic therapy2 and education efforts that emphasize the urgency of treating acute stroke.3 In light of these advances, it is essential that the pathophysiological events that complicate early cerebral reperfusion be elucidated.

Early models of stroke involved inducement of global cerebral ischemia by reduction of carotid blood flow.4 However, this protocol did not reflect the true nature of stroke, because the majority of human ischemic strokes are embolic in origin and result in focal, rather than global, cerebral infarction.1 Thus, animal models of middle cerebral artery occlusion and reperfusion (MCAO-R) were developed as a more appropriate method to examine focal embolic stroke in humans.5 Using this model, researchers found that stroke and reperfusion results in a complex, inflammatory response, mediated in part by leukocytes.6 Using histologic techniques, a number of studies demonstrated that, after hours or days, leukocytes contribute to additional cerebral injury after MCAO.7,8 Although information from these studies is invalu-
able, the studies yield little information about the behavior of leukocytes in the cerebral vasculature during the first hours of reperfusion after stroke. This information is essential for optimization of the timing of initiation of anti-inflammatory therapy.

In response to inflammatory signals from ischemic and reperfused tissue, leukocytes initially accumulate in the vasculature by adhering to the vascular endothelium and plugging capillaries.9,10 While still in the vasculature, activated leukocytes release toxic mediators that damage the nearby vascular and surrounding parenchymal cells. Leukocytes can also adversely affect blood rheology11,12 and promote thrombosis.13 These effects can be rapid. Thus, leukocytes may participate in cerebral-tissue injury during the early minutes of reperfusion. Using in vivo fluorescent microscopy techniques, investigations in vital organs (for example, the heart10 and brain14) indicate that leukocytes accumulate in the microcirculation within minutes after reperfusion that follows ischemia. However, the patterns and mechanisms of leukocyte accumulation and the relationship of that accumulation to rheological changes in cerebral microvessels during the first minutes of reperfusion after MCAO are unclear. Elucidation of this information is important to develop therapies aimed at reduction of early leukocyte-mediated inflammatory response initiated by cerebral ischemia and reperfusion.

The purpose of the present study was to use a physiologically stable in vivo model of direct observation of the cerebral microcirculation after MCAO-R to characterize the patterns of leukocyte accumulation and the hemodynamic changes in the cerebral microcirculation after MCAO-R. We found that coupling MCAO-R with dual-labeling fluorescence microscopy techniques provides a unique and well-controlled experimental method for examining the relationship between leukocyte accumulation and blood shear rate in the cerebral microcirculation after stroke. We observed that 2 hours of MCAO and 1 hour of reperfusion resulted in significant leukocyte rolling and adhesion to cerebral venules. In addition, we observed that leukocyte adhesion to venules was associated with a significant reduction in blood shear rate after MCAO-R. The experimental model described in the present study is useful for examination of the mechanisms of blood cell–endothelium interactions and the rheological events associated with acute stroke and reperfusion.

Materials and Methods

Animal Preparation

All experiments were conducted according to the guidelines issued by the Institutional Animal Care and Use Committee and were in compliance with the NIH Guide for the Care and Use of Laboratory Animals. Anesthesia was induced in male Sprague-Dawley rats (250 to 350 g) with 3% halothane and 1 mL/min oxygen; the rats were intubated (polyethylene tubing; PE-200) and anesthesia continued for the duration of the experiment with artificial ventilation (Harvard rodent ventilator, model 683) by use of 0.8 mL of N2O2, 0.6 mL of O2, and 0.5% to 1.5% halothane. Tidal volume was adjusted as needed to maintain physiological blood gases. Body temperature was continuously monitored and maintained at 37°C with a feedback-controlled heating blanket and rectal probe (Gaymar T-pump, Kent Scientific). Tail artery and vein catheters (PE-10 heat-connected to PE-50) were inserted for blood pressure monitoring and blood sampling for gases (Radiometer, ABL5) and for administration of drugs, respectively. Intravenous vecuronium bromide (2 mg/mL) was administered continuously (1.2 mL/h) after tail catheter placement.15,16

Middle Cerebral Artery Occlusion and Reperfusion

MCAO was induced by the intraluminal filament method as described by Z ea Longa et al.17 The right common carotid artery was exposed through a midline incision and separation of the omohyoid muscle. The external carotid artery (ECA) was dissected from surrounding fascia and nerves, and the occipital artery and superior thyroid artery branches of the ECA were cauterized. The ECA was then tubed with 4-0 silk and cauterized. The proximal branch of the internal carotid artery (ICA) was dissected free to visualize the correct placement of the filament into the ICA. A microvascular clamp was applied to the external carotid stub, and a small hole was cut above the clip with microdissecting scissors. A 30-mm segment of nylon filament (3-0 nylon; Harvard) was prepared by rounding the tip to approximately 0.25 mm in diameter with a cautery. The filament was placed in the ECA, and a silk suture (6-0 silk) was tied around the vessel and the filament to prevent bleeding. The microvascular clamp was removed, and the filament was advanced 18 mm into the ICA, or until a slight bending of the filament was visualized. The neck incision was then closed. After 2 hours of ischemia, the neck incision was reopened and the intraluminal filament was withdrawn into the ECA. The incision was closed, and the animal was immediately prepared for direct observation of the microcirculation.

Cranial-Window Preparation

After 90 minutes of ischemia, the rat was placed in a stereotactic frame and a 4×6-mm craniotomy was performed over the right temporal parietal cortex with a hand-held drill. Saline-soaked gauze was placed over the craniotomy until after the intraluminal filament was withdrawn. Immediately after reperfusion, an edge of the dura was carefully lifted and then pierced and cut with a 26-gauge needle. Care was taken not to touch the surface of the brain during removal of the dura. The dura was retracted away from the opening, and the surface of the brain was immediately and continuously superfused with artificial cerebrospinal fluid (CSF).17–20 To ensure that the surface of the brain was continuously immersed in warm, aerated artificial CSF, a dam with a drainage outlet was fashioned around the craniotomy with the skin of the scalp. Polyethylene tubing (PE-50), connected to the artificial CSF setup, was positioned over the open window. Artificial CSF was made on the day of the experiment according to the methods of Sadoshima et al.17 The following was added to 1 L of deionized water (mmol/L): KCl 2.9, MgCl2 1.4, CaCl2 1.9, NaCl 132, NaHCO3 19, urea 6.7, and glucose 3.7. Artificial CSF was kept at 37°C with a water bath and aerated with 7% CO2, 7% N2, and 86% O2.

Direct Observation of the Microcirculation

During reperfusion, the cerebral microcirculation was directly visualized with a fluorescence microscope (Zeiss MPS) equipped with appropriate filter sets necessary for visualization of the fluorescent dyes FITC and rhodamine. Several days before the experiment, 5% FITC-albumin was injected into the anterior chamber of the eye. This concentration of FITC-albumin does not activate leukocytes.18,22 The use of FITC-albumin provides a bright contrast in the microvessels so they can be accurately identified and measured. FITC-albumin preparation was modified from the methods of Lee and McDonagh18 and McDonagh and Williams.18,22 FITC (6.25 mg; Sigma Chemical Co) and albumin (1.25 g; Sigma) were stirred in cold bicarbonate buffer (25 mL) overnight. To remove unbound FITC from the solution, the FITC-albumin was eluted through a 50 mL Sephadex (G-25M; Amersham Pharmacia Biotech) column and then ultracentrifuged (Amicon Centriprep-30; Millipore). For visualization of the cerebral microcirculation, 1 mL of FITC-albumin was injected intra-arterially immediately before data collection.18 For visualization of leukocytes
in the microcirculation, 500 µL of freshly prepared 0.1% rhodamine 6G (Sigma) was injected intra-arterially 10 minutes before data collection was initiated. Rhodamine selectively labels leukocytes and platelets in vivo and, at this concentration, does not activate leukocytes.23,24 For data collection, the pial microcirculation was initially brought into focus using a ×10 objective. A ×32 objective was then used to visualize single microvessels. With the ×32 objective, the specimen-to-monitor magnification was ×780. By use of the FITC filter, a capillary network, arteriole, or venule was identified. While remaining on a single vessel, the rhodamine filter was then used to visualize leukocytes within the same vessel. Images were recorded on ½-in videotape recorder (Mitsubishi U82).

After 15, 30, and 60 minutes of reperfusion, cerebral capillaries, arterioles (30 to 70 µm), and venules (20 to 90 µm) were videotaped. At least 6 to 8 capillaries, arterioles, and venules were randomly selected and recorded at each time point. Leukocyte accumulation was assessed on video playback by counting the number of leukocytes that rolled a distance of ≥3 video frames. The accumulation in capillaries was expressed as the number of leukocytes per 105 m in a venule past a defined reference point were counted.26 The accumulation in microvessels was expressed as the number of leukocytes per 5 × 10^3 µm.27 For each venule, 3 to 6 leukocytes were counted. The number of leukocytes rolling in venules and venular shear rates were also assessed during the entire 60 minutes of reperfusion. For leukocyte rolling, the number of leukocytes that rolled a distance of 100 µm in a venule past a defined reference point were counted.28

Calculation of shear rate requires a measurement of microvascular blood velocity and vessel diameter.27–29 Centerline velocity of leukocytes (V_{wbc}) was measured by use of the labeled leukocytes as natural markers of blood flow as previously described.28–30 V_{wbc} values closely approximate the velocities of platelets and erythrocytes.31 During video playback, V_{wbc} (in µm/s) was measured as the distance the leading edge of a leukocyte traveled in ≥3 video frames. V_{wbc} was then calculated as 30 frames/s times the distance traveled (in µm) divided by the number of frames. For each venule, 3 to 6 leukocyte velocities were measured and averaged. Shear rate was subsequently calculated on the basis of Poiseuille flow as

$$\frac{8(V_{wbc}/D)}{},$$

where V_{wbc} is center velocity/1.6 and D is venule diameter.28–29,32

**Experimental Protocol**

Two groups of rats were studied: a sham-operated control group (n=7) and a group subjected to 2 hours of MCAO and 1 hour of reperfusion (MCAO-R) (n=7). The sham control group underwent the same procedures as the experimental group except for placement of the filament.3 Animals were intubated and ventilated and tail catheters were placed. MCAO was then performed. After 90 minutes of ischemia, the cranial-window preparation was initiated. After 2 hours of occlusion, the filament was removed. The cranial-window preparation was then finished by removing the dura to expose the pial microcirculation. Rhodamine was administered 10 minutes before reperfusion. FITC-albumin was administered immediately before data collection. Leukocyte accumulation, rolling, and velocity were assessed at 15, 30, and 60 minutes of reperfusion and at the same time points after sham operation. Arterial blood gases, CSF gases, and blood pressure were monitored before and during ischemia and during reperfusion. Animals that demonstrated nonphysiological arterial blood gases or artificial CSF were excluded from the study. A detailed schematic diagram of the experimental model is illustrated in Figure 1.

**Statistical Analysis**

Video analysis of leukocyte accumulation was performed in a blinded fashion. Data were collected and tabulated on computer spreadsheets (Microsoft Excel, version 7.0). Summary data were expressed as mean±SEM. Comparisons between groups were made by Student’s t test or repeated-measures ANOVA and Fisher’s post hoc analysis to determine significant differences (GBStat, version 6.5). P≤0.05 was considered statistically significant.

**Results**

**Experimental Model**

In the present study, we report a rat model of stroke and reperfusion that uses in vivo fluorescence microscopy, which provides direct observation of the cerebral microcirculation. With this model, we provide new information regarding early in vivo leukocyte-vascular interactions and rheological changes in a clinically applicable model of stroke. As Table 1 indicates, key physiological parameters of animals undergoing MCAO and direct observation of the cerebral microcirculation were maintained within physiological ranges before ischemia and during reperfusion. Sham-operated controls and MCAO-R rats did not statistically differ with respect to blood gases, CSF gases, blood pressure, body temperature, or body weight.

With the use of carefully prepared FITC-albumin, a sensitive TV camera, and a specimen-to-monitor magnification of ×780, we were able to resolve capillaries,
venules, and arterioles in the pial microcirculation of the cortex, as demonstrated in Figure 2.

Leukocyte Adhesion to Venules
Leukocytes in the microcirculation are easily visualized and quantified after rhodamine injection. Rhodamine also stains platelets. Leukocytes and platelets are readily distinguished from one another by size; platelets are approximately 1/8 the size of a leukocyte. We observed significant leukocyte accumulation in the cerebral microcirculation during early reperfusion after MCAO, which occurred primarily in venules. Leukocyte adhesion to cerebral venules was significantly increased at 15, 30, and 60 minutes of reperfusion after MCAO (n = 110 venules) compared with sham controls (n = 60 venules; \( P < 0.01 \); Figures 3 and 4). An approximate 4-fold to 5-fold increase occurred at 15 and 30 minutes of reperfusion and a 25-fold increase after 1 hour of reperfusion. When leukocyte accumulation in venules of control animals occurred, it was often at turns or bifurcations in the venule. In addition to the above observations, a greater amount of FITC-albumin leakage appeared to be present from venules of ischemic-reperfused animals compared with controls, but we did not quantify this observation. This observation has been reported by others.\(^{33}\)

Leukocyte Adhesion to Arterioles
Arterioles were easily distinguished from venules because of their smaller diameter, straight-edged appearance, and greater blood velocity (Figure 2C). Leukocytes were rarely observed to adhere to arterioles of control animals. Leukocyte adhesion to arterioles increased after 30 (\( P < 0.05 \)) but not at 15 or 60 minutes of reperfusion (Figure 5).

Leukocyte Trapping in Capillaries
With the use of FITC-albumin, pial capillaries were clearly visible (Figure 2C). Few leukocytes were trapped in cerebral capillaries in control animals. Leukocyte trapping in capillaries was greater in the MCAO-R group than in controls after 15 (\( P = \text{NS} \)) and 30 minutes (\( P < 0.05 \)) of reperfusion. However, this trend did not persist after 1 hour of reperfusion (Figure 6).

### Table 1. Physiological Parameters

<table>
<thead>
<tr>
<th></th>
<th>Sham Control</th>
<th>MCAO-R</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preischemia ABGs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.39±0.03</td>
<td>7.40±0.03</td>
</tr>
<tr>
<td>( P_{CO_2} )</td>
<td>42.3±6.61</td>
<td>36.6±1.53</td>
</tr>
<tr>
<td>( P_O_2 )</td>
<td>123.2±4.14</td>
<td>94.7±4.80</td>
</tr>
<tr>
<td>( O_2 ) saturation</td>
<td>98.5±0.07</td>
<td>97.06±0.71</td>
</tr>
<tr>
<td><strong>Artificial CSF</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>36.75±0.25</td>
<td>37.00±0.00</td>
</tr>
<tr>
<td>pH</td>
<td>7.33±0.09</td>
<td>7.44±0.03</td>
</tr>
<tr>
<td>( P_{CO_2} )</td>
<td>33.72±4.75</td>
<td>26.91±1.60</td>
</tr>
<tr>
<td>( P_O_2 )</td>
<td>117.33±9.22</td>
<td>112.40±0.95</td>
</tr>
<tr>
<td>( O_2 ) saturation</td>
<td>97.26±0.73</td>
<td>98.34±0.15</td>
</tr>
<tr>
<td><strong>MAP, mm Hg</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemia</td>
<td>127.0</td>
<td>129.28±6.16</td>
</tr>
<tr>
<td>Reperfusion</td>
<td>142.33±1.45</td>
<td>118.58±7.22</td>
</tr>
<tr>
<td><strong>Body temperature, °C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemia</td>
<td>37.5</td>
<td>37.33±0.27</td>
</tr>
<tr>
<td>Reperfusion</td>
<td>37.5</td>
<td>37.00±0.58</td>
</tr>
<tr>
<td><strong>Weight, g</strong></td>
<td>323.6±8.05</td>
<td>303.50±13.03</td>
</tr>
</tbody>
</table>

ABG indicates arterial blood gas; MAP, mean arterial blood pressure.
Leukocyte Rolling in Venules

Leukocyte rolling in venules was infrequently observed in venules of control animals. Occasionally, platelet adherence and rolling was observed in control venules, but this occurrence did not appear to be associated with leukocyte adherence to the platelets or endothelium. In contrast, a significant increase in the number of rolling leukocytes occurred in venules at 15, 30, and 60 minutes of reperfusion compared with sham controls ($P \leq 0.01$; Figure 7). In addition to the above findings, we often observed leukocyte aggregates that were adhered to, or rolling on, venules in animals subjected to MCAO-R. We also observed platelet-venule adherence and leukocyte-platelet aggregates in ischemic-reperfused animals (data not quantified).

Shear Rates

We measured blood-cell velocities and calculated shear rates in cerebral venules during 1 hour of reperfusion after MCAO. As seen in Table 2, a significant amount of heterogeneity of blood flow existed in both groups, as evidenced by the wide range of velocities and shear rates. We observed a decrease in shear rate over time in the control groups. Averaged over the entire reperfusion period, mean velocity and shear rate were significantly lower in venules of the MCAO-R group ($n=42$) compared with controls ($n=33$) (velocity, $900.9 \pm 111.3$ versus $1626.7 \pm 237.8$ mm/s, $P \leq 0.05$; shear rate, $82.7 \pm 12$ versus $188.9 \pm 29.6$ s$^{-1}$, $P \leq 0.01$, respectively).
Discussion

Studies that examine in vivo intravascular leukocyte behavior and rheological changes after stroke may yield additional information with regard to the timing, magnitude, and relative importance of the postischemic inflammatory response. Using a model that combines a clinically relevant model of stroke with direct observations of the cerebral microcirculation, we report patterns of early leukocyte interactions with the vasculature and hemodynamic changes in the brain during reperfusion after stroke. To our knowledge, the present study is the first to use a dual fluorochrome technique (FITC-albumin and rhodamine) to examine both the cellular-adhesion events and hemodynamic changes in the cerebral microvessels after MCAO.

Leukocytes and Cerebral Reperfusion Injury

The blood-leukocyte response is one component of the acute inflammatory cascade that is initiated during cerebral ischemia and reperfusion. It is well documented that leukocytes accumulate in the brain after stroke and that they contribute to reperfusion injury. Early studies that used histologic analysis revealed leukocyte accumulation in ischemic brain tissue as early as 1 hour after reperfusion that peaked 24 to 48 hours after reperfusion. Convincing evidence that leukocytes contribute to cerebral reperfusion injury comes from studies that use antileukocyte interventions delivered during reperfusion. For example, Chopp et al demonstrated that administration of an antibody against the leukocyte antigen Mac-1 1 hour after onset of reperfusion significantly reduced the size of cerebral infarction and extent of leukocyte infiltration after 2 hours of MCAO. Similar strategies that used antibodies against leukocyte-adhesion molecule CD11b, endothelial cell–adhesion molecules intercellular adhesion molecule-1 (ICAM-1), and E-selectin demonstrated similar reduction in the size of cerebral infarction and reduction of leukocyte accumulation. Blockage of leukocyte accumulation before or after cerebral reperfusion has been associated with improvements in electrophysiological function, edema, neurological function, and the size of infarction. Several studies demonstrated that leukocytes are activated during experimental stroke and reperfusion. While still in the vasculature, activated leukocytes may contribute to additional cellular injury by releasing toxic mediators, by contributing to hypoperfusion, and by potentially exaggerating thrombotic processes.

Early Vascular Leukocyte Accumulation After Stroke

Leukocyte Adhesion and Rolling in Venules

We report a significant leukocyte accumulation in cerebral venules after 15 minutes of reperfusion and persisting for 60 minutes of reperfusion. Other in vivo studies have examined the initial inflammatory event of leukocyte accumulation in the microcirculation after global cerebral ischemia, which was induced by asphyxia or bilateral carotid occlusion. Using direct observation techniques, these investigators reported significant leukocyte accumulation in postcapillary venules during early reperfusion. However, in contrast to our findings, Hudetz et al and Dirnagl et al found that leukocyte adhesion returned to preischemic baseline after 60 minutes of reperfusion. Similar to our findings, Gidday et al reported a significant and persistent increase in leukocyte accumulation in piglet cerebral venules during 2 hours of reperfusion after 9 minutes of asphyxia or 10 minutes of global ischemia. Using a combined MCAO and fluorescence microscopy technique, Ishikawa and colleagues recently reported the effects of hypothermia on leukocyte accumulation in the microcirculation after 1 hour of focal stroke. Similar to our findings, they reported a significant leukocyte accumulation in venules of normothermic animals after as few as 30 minutes of reperfusion, which persisted for 3 hours of reperfusion.

In addition to firm leukocyte adhesion to venules, we observed a significant increase in the number of rolling leukocytes after focal stroke and reperfusion. Previous studies in other microvascular beds have characterized the cellular mechanisms involved in leukocyte rolling on the endothelium during reperfusion after ischemia. The selectin family of adhesion molecules (L-, P-, and E-selectin) is responsible for
the initial tethering and rolling of leukocytes to the endothelium; these molecules are upregulated during ischemia and reperfusion. An increase in leukocyte rolling may subsequently lead to an increase in firm adhesion to the endothelium, which is mediated by the leukocyte adhesion molecule CD11b/CD18 (integrin) and its endothelial ligand, ICAM-1.49 Our findings that both leukocyte rolling and adhesion are significantly increased suggest that both selectin and integrin adhesion molecules are active cellular participants in MCAO-R.

### Leukocyte Adhesion to Arterioles

We observed a significant increase in leukocyte adhesion to arterioles only after 30 minutes of reperfusion. Hudetz et al15 and Dirnagl et al16 did not observe significant leukocyte adherence in arterioles after global ischemia. Ishikawa and colleagues14 reported increases in leukocyte adherence to arterial endothelium throughout a 3-hour period of reperfusion after MCAO. We suspect that model differences may explain the differences in these findings. In our preparation, early (after 15 minutes of reperfusion) or persistent (after 60 minutes of reperfusion) significant arteriole leukocyte accumulation most often indicated that arterial blood gases or artificial CSF gases were not in physiological range. In these cases, the animals were excluded from the study.

### Leukocyte Trapping in Capillaries

In the present study, leukocyte plugging in capillaries of the pial microcirculation of rats after MCAO-R was not significantly different than in controls. This observation is consistent with the findings of others.16,50,51 In contrast to capillaries in the brain, capillaries in the heart10,28 and lung,52 are smaller in diameter (5 μm), and leukocyte plugging is significant in these organs during ischemia-reperfusion and inflammation. The diameter of a typical cerebral capillary is approximately 9 μm, which may be large enough to allow passage of stiff, activated leukocytes. Although leukocyte trapping in postischemic cerebral capillaries is not a consistent observation, it has been postulated by some to be a mechanism contributing to the hypoperfusion state during reperfusion.11,12,45 In the present study, we observed a decrease in postischemic perfusion (discussed below). However, our present findings indicate that leukocyte capillary plugging was not responsible for the decrease in postischemic blood flow we observed.

### Hemodynamic Changes After Stroke and Venular Shear Rate

We report for the first time the hemodynamic changes in the microcirculation after stroke and early reperfusion by use of MCAO and in vivo fluorescence microscopy techniques. In the present study, we observed a significant decrease in shear rate after stroke and reperfusion. Blood velocities in the cerebral venules of control animals in the present study (mean velocity, 1.7 mm/s) were similar to those of Ma et al,53 who reported mean velocities of 1.5 to 2.8 mm/s in 60- to 100-μm venules of rats. The observation of a reduction in blood flow after cerebral ischemia (hypoperfusion) has been reported by a number of investigators who used microvascular patency techniques11,12,45 or Doppler flowmetry.16 Without attempting to locate the exact vascular sites of accumulation, Hallenbeck et al15 in an early study described leukocyte accumulation in regions of ischemic-reperfused brains that demonstrated low blood flow or heterogeneous blood flow, as measured by autoradiography. We previously demonstrated that in the postischemic reperfused heart low shear rates resulted in significant leukocyte accumulation in coronary venules. We extend this observation and that of Hallenbeck et al11 with our present finding that significant leukocyte accumulation occurs in postischemic-reperfused cerebral venules that demonstrate lower shear rates. One explanation for the persistent low shear rates during reperfusion is the presence of leukocytes plugged in capillaries (see below). However, the contribution of leukocyte plugging to low shear rates after cerebral ischemia remains controversial. Alternatively, several investigators suggest that after cerebral ischemia-reperfusion, activated leukocytes and damaged brain cells may release inflammatory mediators that promote coagulation and vasoconstriction, which leads to the hypoperfusion state.16,50,54,55 The use of FITC-albumin clearly demarcates vessel margins, making this model ideally suited for studies that examine vasogenic reactivity and vascular permeability after focal stroke.

We observed that venular shear rate decreased over time in the control group. It is well documented that halothane is a dilator of cerebral arteries.56,57 The effects of volatile anesthetics on the vasoreactivity of venules is less clear. In the present study, shear rate was measured in venules. Because shear rate is inversely related to vessel diameter, it is possible that prolonged exposure to halothane resulted in progressive venodilation and, subsequently, reduced shear rate. This possibility is supported by the observation that mean venule diameter tended to increase over time in the sham control group. Shear rates in the MCAO-R group remained significantly lower throughout reperfusion despite the reduction in shear rates in the control group. Note that reduced shear rate

### TABLE 2. Velocities and Shear Rates in Venules After MCAO-R

<table>
<thead>
<tr>
<th>Reperfusion Time, min</th>
<th>Group</th>
<th>N</th>
<th>Diameter, μm</th>
<th>$v_{ave}$, μm/s</th>
<th>Shear Rate, s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Sham</td>
<td>14</td>
<td>44.6±4.2 (30–150)</td>
<td>2341±486 (3000–6000)</td>
<td>274.1±60.1 (37.5–750)</td>
</tr>
<tr>
<td>MCAO</td>
<td>4</td>
<td>85±22.2 (70–150)</td>
<td>1496±371 (585–2100)</td>
<td>90.6±41.8 (41.8–210)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>Sham</td>
<td>15</td>
<td>50±4.2 (25–80)</td>
<td>1179.3±189.9 (314–2629)</td>
<td>132.3±23.6 (31.7–328.1)</td>
</tr>
<tr>
<td>MCAO</td>
<td>22</td>
<td>58±4.1 (20–90)</td>
<td>905.4±145.2 (187.5–1650)</td>
<td>90.9±19.2 (7.5–412.5)</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>Sham</td>
<td>4</td>
<td>52.5±12.3 (20–80)</td>
<td>862.6±145.8 (562.5–1125)</td>
<td>115.4±55.4 (51.1–281.3)</td>
</tr>
<tr>
<td>MCAO</td>
<td>16</td>
<td>60.3±4.5 (25–90)</td>
<td>745.9±184 (75–1200)</td>
<td>69.5±14.8 (17.6–214)</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM and (range).
was associated with an increase in leukocyte rolling and adhesion only in the MCAO-R group. This indicates that the combined events of ischemia-reperfusion and low shear rate, but not reduced shear alone, promote significant leukocyte accumulation.

Reduced shear rate in ischemic venules promotes leukocyte rolling and subsequent firm adhesion to the endothelium.29 We observed a significant increase in both leukocyte rolling and leukocyte adhesion in the presence of low blood shear rate. Good evidence exists that under conditions of low shear rate, both selectin and integrin adhesion interactions contribute to leukocyte accumulation.25,58

Summary

In conclusion, in the present study we have extended the observations of others that leukocytes are significant participants in cerebral ischemia-reperfusion. We found that 2 hours of MCAO and 1 hour of reperfusion results in significant leukocyte rolling and adhesion to cerebral venules. Leukocyte trapping in capillaries and adhesion to arterioles after MCAO-R tended to increase compared with controls, but to a lesser extent than the significant increase in leukocyte adhesion in venules. We also report for the first time that shear rate is significantly reduced in the cerebral microcirculation after focal stroke and reperfusion. We describe an in vivo stroke model that combines the filament method of MCAO-R with dual-labeling in vivo fluorescence microscopy techniques for direct observation of the cerebral microcirculation. The present model can be used to accurately examine vascular reactivity and behavior of blood cells and in the cerebral microcirculation after stroke. Information from these and similar experiments may contribute to our understanding of the early inflammatory response in the brain after stroke and reperfusion.

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Ritter et al have used a well-established model of local ischemia-reperfusion to study the location and time course of leukocyte accumulation in vessels overlying and within the superficial cortex. This is important because leukocyte rolling and adhesion are important pathogenetic factors in the evolution of damage in infarction. Provided that these observations are mimicked by events in humans, this model could serve as a means of testing postischemic therapies for efficacy and for relating efficacy to effects on leukocyte behavior.

However, all workers who use fluorescent tags for in vivo studies must be absolutely certain that the experimental technique does not injure the endothelium. An extensive literature, much of it by this writer, shows that “light/dye” injuries can produce endothelial injury with consequent adhesion of circulating cells to the injured area. The present writer’s studies concerned platelets. In the work of Ritter et al, the fluorescent tag labeled both platelets and leukocytes. It seems odd that the article does not mention platelet adhesion in the same areas as those said to contain leukocytes. Moreover, irrespective of the nature of the adhering cells, the questions remain of whether the light/dye technique injured the vessels and whether the reduction in flow during ischemia superimposed additional injury. These questions are particularly troublesome because the controls were not free of leukocyte accumulation at venular bifurcations. The magnitude of this finding is not sufficiently described. Also, some degree of dye leakage is described from venules in control animals. In my experience with albumin-bound fluorescein and mice, no leakage occurred from controls exposed only for seconds to light. Leakage occurred after longer exposures, especially those that induced platelet aggregation. It is important to see whether light/dye produced damage in the present study, by performing electron microscopic studies of vessels and whether the reduction in flow during ischemia was noted or the first accumulation of leukocytes was seen.


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