Effects of Moderate Hypothermia on Leukocyte-Endothelium Interaction in the Rat Pial Microvasculature After Transient Middle Cerebral Artery Occlusion

Mami Ishikawa, MD; Eliichi Sekizuka, MD; Shuzo Sato, MD; Noriyuki Yamaguchi, MD; Joji Inamasu, MD; Helmut Bertalanffy, MD; Takeshi Kawase, MD

Background and Purpose—It has been demonstrated that moderate hypothermia attenuates brain damage, but the mechanism whereby this is achieved has not been clearly shown. Recently, the role of leukocytes as mediators of secondary brain damage after brain ischemia has been discussed. The aim of this study is to examine the effects of moderate hypothermia on leukocyte-endothelium interaction in the rat pial microvasculature after transient middle cerebral artery occlusion (MCAO).

Methods—Rhodamine 6G–labeled leukocytes in brain surface were visualized with intravital fluorescence videomicroscopy through a closed cranial window. We analyzed the number of leukocytes adhering to the venular and arteriolar endothelium before ischemic insult and up to 3 hours after reperfusion. Rats were divided into 4 experimental groups. Group I (n=6) consisted of sham-operated animals. Groups II (n=6) and III (n=6) received left MCAO for 1 hour under normothermia (36°C to 37°C, group II) and under moderate hypothermia (30°C to 32°C, group III). Group IV (n=4) received left common carotid artery occlusion for 1 hour under normothermia.

Results—The number of adhering leukocytes in venules in groups II and IV increased significantly (P<0.001) after reperfusion compared with the group I, but that in group III did not increase significantly (P>0.05). The number of adhering leukocytes in arterioles in group II increased significantly (P<0.01) compared with the other groups, although the adhering leukocytes were not as numerous as those seen in venules.

Conclusions—It is demonstrated that hypothermia attenuates adhering leukocytes in venules and arterioles after reperfusion of MCAO. The inhibition of the leukocyte function may be an important factor in the neuroprotective effect of hypothermia. (Stroke. 1999;30:1679-1686.)

Key Words: hypothermia ■ leukocytes ■ middle cerebral artery occlusion ■ reperfusion injury

The pathogenesis of secondary brain damage after events such as cerebral ischemia and traumatic brain injury has been examined, and the role of leukocytes as inflammatory mediators of secondary injury has been recognized in the brain as well as other main body organs. In vivo evidence of adherent leukocytes induced by global cerebral ischemia or traumatic brain injury has been provided early after brain damage. However, hypothermia has been shown to have a neuroprotective influence on brain damage experimentally and clinically, and it was reported histopathologically in the brain after middle cerebral artery occlusion (MCAO) that the inflammatory response was reduced, and that the expression of intercellular adhesion molecule 1 (ICAM-1) protein was attenuated by intras ischemic hypothermia.

See Editorial Comment, page 1685

The goal of the present study was to observe the in vivo behavior of leukocytes in the pial venules and arterioles with the use of the closed cranial window and the MCAO model by the intraluminal filament technique and to assess the effects of moderate hypothermia on the phenomenon of leukocyte adhesion after reperfusion to examine whether inhibition of leukocyte function is an important factor in the neuroprotective effect of hypothermia.

Materials and Methods

Animal Preparations

Experiments were performed on male Wistar rats weighing ~250 g. The animals were anesthetized with an intraperitoneal injection of α-chloralose (60 mg/kg) and urethane (600 mg/kg), and lidocaine (1%) was used for local anesthesia. All rats were tracheostomized
and mechanically ventilated with room air (CWE Inc, SAR-830). The femoral artery and vein were cannulated for monitoring mean arterial blood pressure (MAPB), sampling arterial blood for blood gas analysis (with pH kept between 7.30 and 7.45), and intravenous administration. Rectal temperature and window (intrathecal) temperature in the cranial window (digital thermometer DT-300, Inter Medical Co Ltd) were monitored and maintained at normal temperature and at moderate hypothermia by a heating pad (ESC4, Omron), an overhead lamp, and a fan.

Experimental brain damage was produced with a rat model of reversible MCAO (the intraluminal filament occlusion model as modified by Memezawa et al\(^\text{24}\)). We also used a 2-piece device consisting of the occluder filament and the guide sheath in this model, which is useful not only for preventing bleeding during insertion of the occluder but also for occluding the middle cerebral artery (MCA) while observing the pial vessels and the motion of the leukocytes in the brain surface. In brief, after the left internal carotid artery was exposed from the carotid bifurcation to the basal cranial, the external carotid artery, the pterygopalatine artery, and the common carotid artery (CCA) (3 mm proximal from the carotid bifurcation) were ligated with silk sutures. After occlusion of the common carotid artery (CCA), the internal carotid artery was occluded by tightening the polyethylene tube. In 4 rats the polyethylene tube (PE-10) was placed loosely around the left CCA for later CCA occlusion (CCAO). After implantation of the cranial window, the guide sheath was advanced 2 mm proximal from the carotid bifurcation (Figure 1). When MCAO was performed after implantation of a cranial window (see below), the 4.0 nylon was further inserted past the MCAO point through the polyethylene tube (PE-10). In 4 rats the polyethylene tube (PE-10) was placed loosely around the left CCA for later CCA occlusion (CCAO). After implantation of the cranial window, the CCA was occluded by tightening the polyethylene tube.

The head of each rat was fixed in a stereotaxic frame (Narishige, SR-6), and the left parietal bone was exposed by a longitudinal midline skin incision. After 3 polyethylene tubes (PE-50; ID, 0.28 mm; OD, 0.61 mm), into which a 4.0 nylon monofilament was inserted, was inserted into the internal carotid artery from the CCA bifurcation, a guide sheath made from a 10-cm-long polyethylene catheter (PE-10; ID, 0.28 mm; OD, 0.965 mm) were fixed on the skull with cyanoacrylate (Alron Alpha), a closed cranial window after a craniectomy was made with the use of a cover glass (12-mm diameter) and quick self-curing acrylic resin (GC Unifast, Japan).\(^\text{23,25}\) Artificial cerebrospinal fluid consisting of Na\(^+\) 147.8 mEq/L, K\(^+\) 3.0 mEq/L, Mg\(^2+\) 2.5 mEq/L, Ca\(^2+\) 2.3 mEq/L, Cl\(^-\) 135.2 mEq/L, HCO\(_3\)\(^-\) 19.61 mEq/L, lactate 1.67 mEq/L, phosphate 1.1 mmol/L, and glucose 3.9 mmol/L was used to fill the space in the cranial window. A probe of a thermometer was inserted into 1 of the 3 polyethylene tubes (PE-50) to measure the window (intrathecal) temperature in the cranial window. There was no cerebrospinal fluid leakage through any space between the probe and the PE-50.

All procedures were performed in accordance with guidelines established by the Animal Care and Use Committee of the Saitama National Hospital.

### Adhesive Leukocytes Assessed With Silicon-Intensified Target Camera

We used an in vivo microscope with a silicon-intensified target (SIT) camera (C2400–80, Hamamatsu Photonics K.K.), a video monitor (Kodak Ektapro 1000), a video timer (VTG-10, FOR-A), and a video recorder (Sony). Leukocytes were labeled with a bolus of 3 μg of rhodamine 6G (absorption peak, 526 nm; emission peak, 555 nm; Sigma) in 1 mL 0.9% saline, which was injected intravenously, followed by a continuous infusion of 1 mL/h at the same concentration. The rhodamine 6G labels circulating polymorphonuclear leukocytes, lymphocytes, monocytes, and platelets but not red blood cells or endothelial cells.\(^\text{10,27}\) Those leukocytes adhering to the vessel wall for 30 seconds per 100-μm-long segment were classed as adhering leukocytes.

### Experimental Protocol

Animals were assigned to 1 of 4 experimental groups as follows.

- **Group I (n=6)** consisted of a sham-operated control group under normothermia without MCAO. Animals were studied for 3 hours, and adhering leukocytes in the venules (45 venular segments) and arterioles (30 arteriolar segments) were determined at 0, 0.5, 1, 2, and 3 hours after preparation.
- **Group II (n=6)** and **III (n=6)** underwent MCAO for 1 hour. Hypothermic rats in group II were maintained at 36°C to 37°C throughout the experiment. Hypothermic rats in group III were maintained at 30°C to 32°C from before the MCAO procedure until the end of the experiment. Adhering leukocytes in the venules (56 venular segments in group II and 66 venular segments in group III) and arterioles (27 arteriolar segments in group II and 31 arteriolar segments in group III) were determined before MCAO and at 0.5, 1, 2, and 3 hours after onset of reperfusion.
- **Group IV (n=4)**, animals underwent CCAO for 1 hour under normothermia. Adhering leukocytes in the venules (43 venular segments) and arterioles (33 arteriolar segments) were determined before CCAO and at 0.5, 1, 2, and 3 hours after onset of reperfusion. Seven to 10 venular segments and 4 to 6 arteriolar segments were randomly chosen per animal in each group.

**Figure 1.** Rectal temperature, window (intrathecal) temperature, and MABP in the 4 groups. There was no significant difference between rectal temperature and cerebrospinal fluid temperature obtained at the same time in each group with repeated-measures ANOVA. There were significant differences in rectal temperature and window (intrathecal) temperature (P<0.001) between group III (hypothermia) and the other groups (groups I, II, and IV) with repeated-measures ANOVA. There was no significant difference in MABP between each group with repeated-measures ANOVA. Values are mean±SD.
Statistical Analysis
The time course of leukocyte adhesion and physiological variables (temperature and MABP) were analyzed with the use of repeated-measures ANOVA. The number of adhering leukocytes at each time period and vessel diameter were analyzed with Scheffé’s F test.

Results
Rectal temperature, window (intrathecal) temperature in the cranial window, and MABP obtained for each group are presented in Figure 1. Vessel diameters of venules and arterioles were not significantly different between each group with Scheffé’s F test ($P > 0.05$).

MCAO During Observation of Pial Vessels
The retrograde flows in the main anastomoses were observed as the 4.0 nylon was further inserted into the polyethylene tube (PE-10). The blood flows in these anastomoses demonstrated retrograde flows immediately after MCAO, and the MCA area of the brain surface continued to be perfused by the anterior cerebral artery and posterior cerebral artery. Arterioles that branched at the anastomoses and penetrated from the brain surface to the brain parenchyma did not demonstrate retrograde flows and maintained normograde flows. These penetrating arterial flows did not stop during MCAO, but in a small number of venules the flows were minimal. Retrograde flows in the anastomoses during MCAO were seen in all rats under normothermia and hypothermia.

Leukocyte Behavior in Venules
Leukocytes adhering to the endothelium of pial venules were observed with the SIT camera (Figure 2).

Adherent Leukocytes After Reperfusion of MCAO
The number of leukocytes adhering to the endothelium of pial venules under normothermia (group II) increased significantly after reperfusion of MCAO compared with the control (group I) by Scheffé’s F test at 0.5, 1, 2, and 3 hours after onset of reperfusion ($P < 0.001$) and by repeated-measures ANOVA ($P < 0.001$) (Figures 3 and 4).

Effect of Hypothermia on Leukocyte Adhesion
The number of leukocyte adhering to the endothelium of pial venules under hypothermia (group III) did not increase significantly after reperfusion of MCAO compared with control (group I) by Scheffé’s F test at 0.5, 1, 2, and 3 hours after onset of reperfusion ($P > 0.05$) and by repeated-measures ANOVA ($P > 0.05$) (Figures 3 and 4). There was a significant difference between the number of adhering leukocytes under normothermia (group II) and hypothermia (group III) by Scheffé’s F test at 0.5, 1, 2, and 3 hours after onset of reperfusion ($P < 0.001$) and by repeated-measures ANOVA ($P < 0.001$) (Figures 2, 3, and 4).

Adherent Leukocytes After Reperfusion of CCAO
The adhering leukocytes were also observed after CCAO (Figures 2 and 3). The number of leukocytes adhering to the endothelium of pial venules (group IV) increased significantly after reperfusion of CCAO compared with the control (group I) by repeated-measures ANOVA ($P = 0.001$) (Figure 4). There was a significant difference between the number of adhering leukocytes after reperfusion of CCAO (group IV) and after reperfusion of MCAO under normothermia (group II) by repeated-measures ANOVA ($P < 0.001$) (Figure 4).

Leukocyte Behavior in Arterioles
Leukocytes adhering to the endothelium of pial arterioles were observed with the SIT camera (Figure 5). The number of adhering leukocytes in arterioles after MCAO under normothermia (group II) was significantly different compared with the other groups (groups I, III, and IV) by repeated-measures ANOVA ($P < 0.01$) (Figure 6), but by Scheffé’s F test there were
Discussion

Combination of a Cranial Window and an Intraluminal Filament MCAO

The combination of the closed cranial window and the noninvasive intraluminal filament MCAO is first described. The change of the microcirculatory dynamics in the brain surface can be monitored at the moment of MCAO and continuously after MCAO, which is the first advantage of this combination. The intraluminal filament MCAO model is widely used as a focal ischemic model. The arterial blood flow did not disappear after MCAO, as shown in the present study, but ischemic damage of the MCA area was recognized later, as demonstrated by hematoxylin and eosin staining and as reported frequently in the literature. (Twenty-four hours after reperfusion, some rats were anesthetized and decapitated and brains were removed for histopathological analysis, but these pathological data were not shown in the present study.) According to Karibe et al,15 cortical blood flow measured by laser-Doppler flowmetry decreased to $\approx 25\%$ of the preocclusion level after MCAO. It was demonstrated by the retrograde flow in brain surface arteries in the MCA area that these residual flows are provided by the anterior cerebral artery and the posterior cerebral artery through anastomoses. Another advantage of this combination is that the retrograde flow in brain surface arteries acts as a signal that adequate MCAO has been achieved. There was no subarachnoid hemorrhage caused by penetration of the wall of the internal carotid artery at the MCA bifurcation, and there was no insufficient insertion of the occluder filament. If the occluder filament was moved backward even slightly in the guide sheath (PE-10), the retrograde flow phenomenon decreased or disappeared and the MCAO became insufficient. However, we definitely induced cerebral infarction in view of the fact that the occluder filament and the guide sheath were fixed with a Kocher clamp at the point where the retrograde flows were observed for 1 hour during MCAO. In the present study we emphasized the investigation of leukocyte adhesion and did not analyze vessel diameter after the MCAO. This combination model is also useful for in vivo examination of vessel reactivity after focal cerebral ischemia with minimal experimental invasion.

Leukocyte Behavior in Venules

It has been recently postulated that leukocytes act as mediators of secondary brain damage in cerebral ischemia.1–5

**Figure 3.** Leukocytes adhering to the venular endothelium in all 4 groups. All data are shown before, 0.5, 1, 2, and 3 hours after reperfusion. There were significant differences ($P<0.001$) between the number of adherent leukocytes in group II (normothermia) and those in the other groups 0.5, 1, 2, and 3 hours after reperfusion with Scheffe’s F test.

significant differences only between group I and group II at 0.5 and 1 hour after onset of reperfusion ($P<0.05$).
Leukocyte accumulation in acute cerebral lesions was demonstrated, and leukocyte adhesion to the endothelium of postcapillary venules was observed before emigration to surrounding tissues. The CD11/CD18 on the leukocytes and ICAM-1 on the endothelium are considered adhesion receptors, and it has been determined that blocking of these adhesion receptors with specific monoclonal antibodies (MoAbs) during or after brain ischemia reduces the size of the area of infarction.3 In the model of MCAO and reperfusion, antileukocytic intervention (anti–polymorphonuclear leukocyte MoAb, anti-CD18/11b MoAb, and anti–ICAM-1 MoAb) reduced leukocyte infiltration in brain tissue and the size of the area of infarction.10 In the present study we showed leukocytes that adhered within 30 minutes after MCAO and CCAO. In this very early phase, constitutively expressed ICAM-1 and the decrease of fluid shear stress may influence leukocyte adhesion to the endothelium. If we identify models in which the expression or action of adhesion molecules can be modulated with anti-CD11b, anti–ICAM-1, or null ICAM-1 mice and then use these models in our experimental observations through the closed cranial window, they will provide some idea regarding the possible mechanisms of the intravascular accumulation of leukocytes during the early postischemic period.28,33,35

Although CCAO on only 1 side did not cause immediate retrograde flow and did not induce brain infarction, the number of adhering leukocytes increased after CCAO. The decreased...
shear stress after CCAO causes the leukocytes to adhere in the vasculature of the brain surface even if actual brain damage has not occurred. If cerebral blood flow stabilizes and there is no brain damage after CCAO, the number of adhering leukocytes may show a decreasing trend over longer follow-up periods, which we did not analyze in the present study.

**Effect of Hypothermia on Leukocyte Adhesion in Venules**

A few investigations of leukocyte function during hypothermia have been performed. Hypothermia attenuated leukocyte migration toward the chemotactic stimulus in vivo and in vitro,35,37 and rolling leukocytes in the postcapillary venules of muscle flap after ischemia-reperfusion injury decreased under hypothermia compared with normothermia.38 In the present in vivo study, it was also demonstrated in the brain microcirculation that moderate hypothermia attenuated leukocyte adhesion after focal ischemia-reperfusion injury. This attenuation of leukocyte adhesion produced by moderate hypothermia is able to reduce brain damage since blocking of the leukocyte adhesion receptors with specific MoAbs during or after brain ischemia reduces infarct size. It has been reported that infarct volume was reduced after MCAO occurred when hypothermia was administered at the onset of ischemia rather than when hypothermia was induced compared with control group and that ICAM-1 expression was attenuated after MCAO by intraschismic hypothermia.23 This suggests that the decrease of leukocyte adhesion induced by moderate hypothermia depends on the attenuated ICAM-1 expression. In the present study, however, the intravascular accumulation of leukocytes during the early postischemic period is temperature sensitive, and it is speculated that the action of the constitutively expressed ICAM-1 is also inhibited by hypothermia before ICAM-1 is induced in the endothelium after reperfusion of MCAO. Moreover, CD11/CD18 expression should also be examined after MCAO during hypothermia.

Karibe et al39 reported that the most substantial reduction of infarct volume after MCAO occurred when hypothermia was induced at the onset of ischemia rather than when hypothermia was induced later. Meanwhile, in reference to the spinal cord, Clark et al40 reported that paraplegia in animals decreased when doxycycline, which inhibited leukocyte function and adhesion in vitro, was administered before ischemia rather than after ischemia. In the present study leukocyte adhesion was also observed in the early phase after MCAO, and it was suppressed by moderate hypothermia. These reports suggest that early leukocyte dynamics after insult affect the cerebral microcirculation and secondary damage and play an important role in infarct size and neurological function, which are decreased by moderate hypothermia.

Blood-brain barrier dysfunction41 as well as leukocyte-endothelium interaction is important as a vascular consequence of cerebral ischemia when one considers the mechanism of the effect of mild or moderate hypothermia on attenuation of brain damage. With the use of both the closed cranial window and intraluminal filament MCAO, the time course of the response of the blood-brain barrier and the relation to leukocyte adhesion should be investigated during normothermia and hypothermia.

**Leukocyte Adhesion in Arterioles**

Rolling and adhering leukocytes and margination of leukocytes can be observed in the venules of most tissues exposed for intravitral microscopy. A few studies reported that leukocyte-endothelium interactions were observed in arterioles but at a lower level compared with venules.42-45 Nazziola and House44 reported that rolling and marginating leukocytes significantly increased under mechanically induced retrograde flow in arterioles compared with normograde flow. In this study the retrograde flow in the vessels was seen in the anastomoses from immediately after MCAO to a few minutes after reperfusion of the MCA. Because of the change of shear rates, adhering leukocytes may be observed in arterioles after MCAO. Iigo et al46 reported that the amount of arteriolar ICAM-1 expression was one tenth that in venules. Nagel et al47 reported that hemodynamic forces increased surface ICAM-1 expression on cultured human umbilical endothelium cells. The role of CD11/CD18 in leukocyte rolling in arterioles is uncertain.48 These adhesive receptors seem to involve arteriolar leukocyte-endothelium interactions, which may vary in each organ as well as venular leukocyte-endothelium interactions. It is possible that leukocytes adhere more easily to the endothelium of arterioles of brain surface than arterioles of other organs, especially under postischemic conditions.

In summary, the combination of the closed cranial window and noninvasive intraluminal filament MCAO is first described. Adhering leukocytes in venules and arterioles after reperfusion of MCAO under moderate hypothermia were statistically significantly attenuated compared with those under normothermia. Inhibition of leukocyte function may be an important factor in the neuroprotective effect of hypothermia. Furthermore, leukocytes adhered to the endothelium of arterioles after reperfusion of MCAO under normothermia but were not as numerous as those seen in venules. The number of adhering leukocytes increased even after reperfusion of CCAO on only 1 side. The behavior of leukocytes after reperfusion of focal ischemia should be further investigated in vivo to demonstrate their role in brain microcirculation.

**References**


They report that moderate hypothermia reduces leukocyte adhesion to the endothelium of rat cerebral blood vessels in the reperfusion phase following focal cerebral ischemia. In view of the important role that neutrophils play in reperfusion injury, these findings suggest that hypothermia may protect the ischemic brain by reducing intravascular adhesion of leukocytes. However, it remains to be demonstrated whether hypothermia also attenuates the infiltration of the ischemic brain by these cells.

The mechanisms of the effect of hypothermia on postischemic leukocyte adhesion remain to be elucidated. Ischemia, either directly or through expression of cytokines, induces adhesion molecules on cerebral vascular cells, which in turn lead to the attachment of leukocytes to cerebral endothelial cells. Then, leukocytes enter the brain parenchyma and are thought to contribute to ischemic injury by producing reactive oxygen species, including nitric oxide. These complex cellular events are driven by a series of molecular changes orchestrated by transcription factors such as the interferon regulatory factor-1 and nuclear factor κB. It is therefore conceivable that hypothermia interferes with some of the molecular signals that initiate neutrophil adhesion to cerebral endothelial cells. Studies addressing the molecular basis of the effects of hypothermia on leukocyte adhesion would be important, because they may provide new strategies to limit postischemic inflammation and improve the outcome of cerebral ischemia.

Although there is ample evidence that postischemic inflammation occurs also in the human brain (see, for example, Reference 5), the role of this cellular reaction in human stroke remains to be defined. A study in which antibodies against the adhesion molecule ICAM-1 were administered to stroke patients failed to show benefit. While the reasons for such failure remain to be determined, increased vascular inflammation induced by the delayed anti-ICAM therapy is a likely possibility (see Reference 7 and its accompanying editorial comment). Therefore, the concept of anti-inflammatory therapy in stroke patients needs to be revisited in the not-too-distant future. In this context, the study of leukocyte adhesion in hypothermia offers the prospect of new therapeutic approaches to decrease the infiltration of the ischemic brain by neutrophils.

Costantino Iadecola, MD, Guest Editor
Laboratory of Cerebrovascular Biology and Stroke Department of Neurology, University of Minnesota Minneapolis, Minnesota

References
Effects of Moderate Hypothermia on Leukocyte-Endothelium Interaction in the Rat Pial Microvasculature After Transient Middle Cerebral Artery Occlusion
Mami Ishikawa, Elichi Sekizuka, Shuzo Sato, Noriyuki Yamaguchi, Joji Inamasu, Helmut Bertalanffy and Takeshi Kawase

*Stroke*. 1999;30:1679-1686
doi: 10.1161/01.STR.30.8.1679

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/30/8/1679

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org/subscriptions/