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 brain1–5 as well as other main body organs.6–9 In vivo evidence of adherent leukocytes induced by global cerebral ischemia10 or traumatic brain injury11 has been provided early after brain damage. However, hypothermia has been shown to have a neuroprotective influence on brain damage experimentally12–18 and clinically,19–21 and it was reported histopathologically in the brain after middle cerebral artery occlusion (MCAO) that the inflammatory response was reduced,13 that the accumulation of leukocytes was reduced,22 and that the expression of intercellular adhesion molecule 1 (ICAM-1) protein was attenuated23 by intracranial hypothermia.

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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 (MABP), 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 al24). 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 cranium, the external carotid artery, the pterygopalatine artery, and the internal carotid artery by a microvascular clip (Sugita type, Mizuho Kagaku Industries) at the pterygopalatine bifurcation, a guide sheath was inserted into the internal carotid artery from the CCA (10-cm-long polyethylene catheter (PE-10; ID, 0.28 mm; OD, 0.61 mm)) at the pterygopalatine bifurcation were ligated with silk sutures. After occlusion of the common carotid artery (CCA) (3 mm proximal from the carotid bifurcation), the guide sheath was advanced 2 mm proximal from the carotid bifurcation to the clipping portion. After removal of the microvascular clip, the guide sheath was advanced 2 to 3 mm from the pterygopalatine bifurcation (Figure 1). When MCAO was performed after implantation of a cranial window (25,26 Artificial cerebrospinal fluid with the use of a cover glass (12-mm diameter) and quick self-curing adhesive (Alon Alpha), a closed cranial window after a craniectomy was made from a 10-cm-long polyethylene catheter (PE-10; 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 ∼1 mm proximal from the carotid bifurcation to the clipping portion. After removal of the microvascular clip, the guide sheath was advanced 2 to 3 mm from the pterygopalatine bifurcation (Figure 1). When MCAO was performed after implantation of a cranial window (25,26 Artificial cerebrospinal fluid with the use of a cover glass (12-mm diameter) and quick self-curing acrylic resin (GC Unifast, Japan),23,24 Artificial cerebrospinal fluid consisting of Na+ 147.8 mM/L, K+ 3.0 mM/L, Mg2+ 2.5 mM/L, Ca2+ 2.3 mM/L, Cl- 135.2 mM/L, HCO3- 19.61 mM/L, lactate 1.67 mM/L, phosphate 1.1 mM/L, and glucose 3.9 mM/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.10,27 Those leukocytes adhering to the vessel wall for 30 seconds per 100-μm-long segment were classified 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.

In groups II (n=6) and III (n=6), animals underwent MCAO for 1 hour. Normothermic 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 (32 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.

In 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.
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 Scheffe’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 Scheffe’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 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 2).

Figure 2. Adhering leukocytes recorded with the SIT camera in venules. 1, Before MCAO. 2, Thirty minutes after reperfusion of MCAO under normothermia. Adhering leukocytes increased after reperfusion (the same venule is shown in 1 and 2). Arrow shows an adhering leukocyte, and arrowhead shows a flowing leukocyte that looks like a shooting star. 3, Three hours after reperfusion of MCAO under hypothermia. Leukocytes adhering to the venular endothelium were not seen. 4, Three hours after reperfusion of CCAO under normothermia. Adhering leukocytes were seen even after CCAO. Bar=50 μm.
significant differences only between group I and group II at 0.5 and 1 hour after onset of reperfusion ($P<0.05$).

**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 preoclusion 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 Scheffé’s F test.](image-url)
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. 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. Activated leukocytes may impair cerebral blood flow by disturbance of microcirculation, may exacerbate endothelial cell injury by releasing hydrolytic enzymes or by producing oxygen free radicals, and may migrate into the ischemic parenchyma to interact with neurons and other supportive cells. There is a possibility that leukocytes in the very early phase of ischemic insult contribute to secondary brain damage. The in vivo behavior of leukocytes in the brain microcirculation was shown, and the adhering leukocytes were observed within 30 minutes after global cerebral ischemia and after traumatic brain injury. 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.

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

Figure 4. Leukocytes adhering to the venular endothelium in all 4 groups. Values are mean±SE, derived from the same data as in Figure 3. There were significant differences with repeated-measures ANOVA between group II (normothermia) and group I (control) (P<0.001), group II and group III (hypothermia) (P<0.001), group II and group IV (CCAO) (P<0.001), and group I and group IV (P=0.001). There was no significant difference between group I and group II with repeated-measures ANOVA (P>0.05). *P<0.001 vs groups I, III, and IV with Scheffé’s F test.

Figure 5. Adhering leukocytes recorded with the SIT camera in arterioles. Left, Adhering leukocytes were observed in arteriole after reperfusion of MCAO under normothermia. Right, Adhering leukocytes in arterioles were not as plentiful as those in venules. A indicates arteriole; V, venules. Bar=50 μm.

Figure 6. Leukocyte adhesion to the arteriolar endothelium in all 4 groups. There were significant differences with repeated-measures ANOVA between group II (normothermia) and group I (control) (P<0.01), group II and group III (hypothermia) (P<0.01), and group II and group IV (CCAO) (P<0.05). *P<0.05 vs group I with Scheffé’s F test. Values are mean±SE.
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, and rolling leukocytes in the postcapillary venules of muscle flap after ischemia-reperfusion injury decreased under hypothermia compared with normothermia. It has been reported that infarct volume was reduced after specific MoAbs during or after brain ischemia reduces infarct size. It has been reported that infarct volume was reduced after MCAO in rats administered anti–ICAM-1 or in null ICAM-1 mice compared with the control group and that ICAM-1 expression was attenuated after MCAO by intraschismic hypothermia. 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 al reported that the marked 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 al reported that paraplegia in animals decreased when doxycycline-induced 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 in rats administered anti–ICAM-1 or in null ICAM-1 mice compared with the control group and that ICAM-1 expression was attenuated after MCAO by intraschismic hypothermia. 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.

**Leukocyte Adhesion in Arterioles**

Rolling and adhering leukocytes and margination of leukocytes can be observed in the venules of most tissues exposed for intravital microscopy. A few studies reported that leukocyte-endothelium interactions were observed in arterioles but at a lower level compared with venules. Nazziola and House 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 al reported that the amount of arteriolar ICAM-1 expression was one tenth that in venules. Nagel et al reported that hemodynamic forces increased surface ICAM-1 expression on cultured human umbilical endothelial cells. The role of CD11/CD18 in leukocyte rolling in arterioles is uncertain. 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. Further examination of the mechanisms concerned and the role of the arteriolar adhesion of leukocytes on brain microcirculation is important and necessary.

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**

Hypothermia remains one of the most powerful neuroprotective strategies in experimental cerebral ischemia. Hypothermia may inhibit glutamate release from ischemic neurons, protect the blood-brain barrier, slow down cellular metabolism, and modify postischemic gene expression, thereby altering the brain’s cellular reaction to injury. In the accompanying article, Ishikawa and colleagues provide a new insight into the mechanisms of the protection afforded by moderate hypothermia.

Ishikawa et al: Effects of Moderate Hypothermia on Leukocyte-Endothelium Interaction

Editorial Comment


hypothermia. 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.6 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

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