Correlation Between Myeloperoxidase-Quantified Neutrophil Accumulation and Ischemic Brain Injury in the Rat

Effects of Neutrophil Depletion

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Background and Purpose  Neutrophils have been implicated in the pathogenesis of ischemia-reperfusion injury. The aim of the present study was to evaluate the correlation between neutrophil infiltration into ischemic tissues and brain injury after transient focal ischemia.

Methods  We evaluated the effects of depletion of circulating neutrophils by administration of an antineutrophil monoclonal antibody (RP3) on brain edema formation, infarct size, and neutrophil infiltration (myeloperoxidase [MPO]-quantified) in rats with 1 hour of middle cerebral artery (MCA) occlusion.

Results  In the cerebral cortex perfused by the anterior cerebral artery (ACA area), there was a significant increase in MPO activity only 24 hours (P<.05) after reperfusion. In the cerebral cortex perfused by the middle cerebral artery (MCA area) and caudate putamen, MPO activity was significantly increased at 12 (MCA area, P<.01; caudate putamen, P<.05), 24 (MCA area, P<.05; caudate putamen, P<.01), and 72 hours (MCA area, P<.01; caudate putamen, P<.05) and returned to near-normal level by 168 hours after reperfusion. Brain MPO activity after transient MCA occlusion correlated well with the appearance of neutrophils. Depletion of neutrophils by RP3 treatment completely inhibited the increase in MPO activity in the ischemic brain after 24 hours of reperfusion. In addition, treatment with RP3 significantly attenuated the postischemic increase in brain water content at 24 hours after reperfusion. RP3 also significantly reduced the size of infarct area.

Conclusions  These results indicate that the increase in brain MPO activity after transient focal ischemia virtually reflects the neutrophil infiltration and that neutrophil infiltration into the ischemic brain is implicated in postischemic brain injury. (Stroke. 1994;25:1469-1475.)

Key Words  cerebral ischemia • neutrophils • neuronal damage • rats

Received December 6, 1993; final revision received February 28, 1994; accepted March 31, 1994.

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The method used to quantify MPO activity from rat brain samples was similar to that recently described by Barone et al.\textsuperscript{11,12} but with minor modifications. The modified MPO assay for brain tissue was conducted as follows. Brain samples were thawed on ice, and wet weight in grams was measured. Each sample was homogenized (1:20, wt/vol) in 5 mmol/L potassium phosphate buffer (pH 6.0, 4°C) using an Ultra-Turrax (Junke & Kunkel Gmb & Co) for three on/off cycles at 5-second intervals and centrifuged at 3000 rpm (30 minutes, 4°C). The supernatant was discarded, and the pellet was washed again as described above. After decanting the supernatant, the pellet was extracted by suspension in 0.5% hexadecltrimethylammonium bromide (HTAB, Sigma Chemical Co) in 50 mmol/L potassium phosphate buffer (pH 6.0, 25°C) for approximately 2 minutes at an original tissue wet weight-to-volume ratio of 1:10. The samples were immediately frozen on dry ice. Three freeze/thaw cycles were then performed with sonication (10 seconds, 25°C, Powersonic Model 50, Powersonic Inc) between cycles. After the last sonication, the samples were incubated at 4°C for 20 minutes and centrifuged at 12 500g (15 minutes, 4°C). MPO activity in the supernatant was assayed as described previously by Bradley et al.\textsuperscript{10} Briefly, 0.1 mL of supernatant was mixed with 2.9 mL of 50 mmol/L potassium phosphate buffer, pH 6.0, containing 0.167 mg/mL o-dianisidine dihydrochloride (Sigma Chemical Co) and 0.0005% hydrogen peroxide (Wako Pure Chemical Co). The change in absorbance at 460 nm was measured with a spectrophotometer (UV-2200, Shimadzu Inc). One unit of MPO activity is defined as that which degrades 1 μmol of peroxide per minute at 25°C.

Brain water content and MPO activity were also measured in complete sham-operated rats.

For histopathology, rats were killed after 6 hours, 12 hours, 24 hours, 3 days, and 7 days of reperfusion after 1 hour of transient MCA occlusion. Brains were perfused transcardially with 100 mL of physiological saline (25°C at a pressure of 100 mm Hg), fixed in formalin, and prepared for embedding in paraffin by routine histological procedures. After embedding, 5-μm coronal sections were cut on a microtome and stained with hematoxylin and eosin. The profile and degree of neutrophil infiltration into the brain were determined and photographed under light microscopy.

The generation of anti-rat neutrophil monoclonal antibody, RP3, was described elsewhere.\textsuperscript{24,25} The ascitic fluid containing a high titer of RP3 was used as an antibody source in the experiment. RP3 selectively reacts and depletes rat neutrophils but not other nucleated blood cells on in vivo administration.\textsuperscript{25} Depletion of neutrophils with RP3 inhibited the late phase response of inflammatory edema in rats.\textsuperscript{25}

To assess the influence of depletion of circulating neutrophils on neutrophil infiltration, brain edema formation, and size of infarct area after ischemia, rats were given 3 mL of the ascitic fluid containing RP3 or vehicle (saline, 3 mL per animal) intraperitoneally, 24 hours before and immediately after ischemic insult. The dose of RP3 chosen in this study is a sufficient dose to induce almost complete depletion of neutrophils from 6 hours to over 24 hours after administration and does not affect other blood cells.\textsuperscript{13,24,25} Brain water content and MPO activity after 24 hours of reperfusion were measured as described above. To measure the size of infarct area, rats were perfused with physiological saline containing 0.2% heparin after 24 hours of reperfusion. Brains were removed, cut into 1-mm coronal sections, and immersed in 2% triphenyltetrazolium chloride (TTC) solution at 37°C for 30 minutes. The colorless areas with TTC staining, which reflect mitochondrial damage, were quantified as infarct areas by an image analyzer system (Kontron M14, Zeiss). The number of neutrophils was counted before RP3 treatment, 24 hours after the first RP3 treatment (immediately before ischemic insult), and 24 hours after the second RP3 treatment (immediately before death). Rats treated with phosphate-buffered saline served as vehicle controls.
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FIG 2. Photomicrographs of cerebral cortex stained with hematoxylin and eosin after 12 (A), 72 (B), and 168 hours (C) of reperfusion following 1 hour of MCA occlusion. A, Neutrophil (small arrowheads) infiltration into the ischemic cortex was observed after 12 hours of reperfusion. B, Neutrophils (small arrowheads) and monocytes (large arrowheads) were observed in the infarcted region after 72 hours of reperfusion. C, After 168 hours of reperfusion, neutrophils disappeared, and a large amount of monocytes (large arrowheads) filled the infarcted region.

Each experiment group consisted of more than 5 animals. All values are presented as mean±SEM. For statistical analysis, Dunnett’s multiple range test and unpaired t test were used.

Results

Sections stained with hematoxylin and eosin under high magnification revealed the infiltration of neutrophils in the ischemic hemisphere after reperfusion, as shown in Fig 2. Few neutrophils were observed in the ischemic hemisphere after 6 hours of reperfusion after 1 hour of MCA occlusion. However, after 12 and 24 hours of reperfusion, a large number of neutrophils were observed in the ischemic hemisphere (Fig 2A). After 72 hours of reperfusion, many neutrophils and monocytes were observed in the infarcted region (Fig 2B). After 168 hours of reperfusion, neutrophils disappeared, and a large number of monocytes were still noticeable in the infarcted region (Fig 2C). Fig 3 illustrates the time course of neutrophil counts in the ischemic hemisphere (per 5-μm section). We could not detect neutrophils in both ischemic and contralateral hemispheres until 6 hours after reperfusion following 1 hour of MCA occlusion. The number of neutrophils in the ischemic hemisphere began to increase from 12 hours (36.2±6.3), reached a peak between 24 hours (60.2±5.8) and 72 hours (68.6±4.7), and almost-infiltrated neutrophils disappeared 168 hours after reperfusion following 1 hour of MCA occlusion.

Fig 4 illustrates the time course of changes in brain MPO activity after reperfusion following 1 hour of MCA occlusion. In the ACA area, there was a significant increase in MPO activity only at 24 hours (0.068±0.013 U/g wet tissue, P<.05) after reperfusion following 1 hour of MCA occlusion (Fig 4A). In the MCA area, MPO activity began to rise from 12 hours (0.040±0.018 U/g wet tissue, P<.01), reached a peak at 24 hours (0.270±0.074 U/g wet tissue, P<.01), and returned to near-normal level (sham-operated, 0.021±0.003 U/g wet tissue) by 168 hours (0.030±0.007 U/g wet tissue) after reperfusion (Fig 4B). In the caudate putamen, MPO activity began to rise from 12 hours (0.075±0.029 U/g wet tissue, P<.05), reached a peak at 72 hours (0.080±0.029 U/g wet tissue, P<.01), and returned to normal level (sham-operated, 0.010±0.002 U/g wet tissue) by 168 hours. In the caudate putamen, we observed a significant increase in MPO activity even at 168 hours (0.027±0.002 U/g wet tissue, P<.01) after reperfusion (Fig 4C). Brain MPO activity after transient MCA occlusion correlated well with the appearance of neutrophils.

Fig 5 illustrates the time course of the changes in brain water content after reperfusion following 1 hour of MCA occlusion. Brain water content continued to rise significantly until 72 hours after reperfusion, and then returned to near-normal level (sham-operated) by 168 hours after reperfusion (Fig 5A, 5B, and 5C). Brain edema formation correlated well with MPO activity, reflecting neutrophil infiltration.
Figs 6 and 7 summarize the effects of the antineutrophil antibody RP3 on the number of circulating neutrophils (Fig 6), MPO activity (Fig 7A), brain water content (Fig 7B), and infarct size (Fig 7C). Depletion of neutrophils was achieved during and after the recirculation period studied (Fig 6), although the number of lymphocytes and monocytes remained unchanged throughout the experimental period (data not shown). RP3 treatment completely inhibited the increase in MPO activity in the ACA area, the MCA area, and the caudate putamen after 24 hours of reperfusion (Fig 7A). In addition, depletion of circulating neutrophils by treatment with RP3 significantly attenuated the postischemic increase in brain water content in the ACA area (P<.01), the MCA area (P<.01), and the caudate putamen (P<.01) compared with vehicle control at 24 hours after reperfusion (Fig 7B). In the ACA area, the increase in water content was completely inhibited by treatment with RP3. In contrast to the ACA area, in the MCA area and the caudate putamen, water contents in the RP3-treated rats were still significantly high (MCA area, P<.01; caudate putamen, P<.01) compared with sham-operated rats. RP3 also significantly reduced the size of infarct area (Fig 7C).

Discussion

The present study indicates that (1) the MPO activity after transient focal ischemia correlated well with the...
appearance of neutrophils and development of brain edema formation and (2) depletion of circulating neutrophils by treatment with RP3 significantly attenuated not only the increase in number of neutrophils into ischemic brain areas but also posts ischemic brain injury. These results strongly suggest that the invasion of neutrophils into the ischemic areas is implicated in the development of post ischemic brain injury.

In the present study, neutrophil accumulation was measured by determining MPO activity. The measurement of MPO as a marker for quantification of infiltrated inflammatory cells has proven to be of value in skin, heart, kidney, and recently also in brain. Barone et al demonstrated that accumulation of neutrophils in focal ischemia can be identified histologically and quantified using an MPO activity assay 24 hours after temporary MCAO with reperfusion. However, the temporal profile of MPO activity and correlation with brain injury have remained undefined. The results of the present study indicate that a 1-hour period of MCAO followed by reperfusion in rats provides sufficient stimulation to induce accumulation of neutrophils in the ischemic brain areas. The increase in neutrophils in ischemic areas occurred slowly, with infiltration beginning between 6 and 12 hours after reperfusion, reaching a peak in MPO activity after 24 hours that persisted until 72 hours and returning to near-normal level by 168 hours after reperfusion. Histological observation of neutrophils correlated well with increases in MPO activity. In contrast, brain water content increased significantly 6 hours after reperfusion, whereas the MPO activity remained unchanged at this time. Brain water content gradually increased, reaching a peak between 24 and 72 hours and returned to near-normal level by 168 hours after reperfusion. These results suggest that an initial increase in brain water content is independent of neutrophil invasion and that a later phase of brain edema is contributed by neutrophils invading ischemic tissues.

Monocytes are granulocytes that also possess MPO activity. In our present study, although a large number of monocytes were still noticeable in the infarcted region 168 hours after reperfusion, MPO activity was at a low level. Bradley et al reported that the level of MPO activity in monocytes is considerably lower than that in neutrophils. The low level of MPO activity at 168 hours after reperfusion indicates that monocytes contribute little to MPO activity in the ischemic brain.

To determine whether MPO activity was actually derived from neutrophils, we examined the effects of depletion of circulating neutrophils by administration of an antineutrophil monoclonal antibody, RP3, on brain MPO activity after 1 hour of MCAO. Depletion of circulating neutrophils with RP3 treatment completely inhibited increases in MPO activity in the ischemic brain and the accumulation of neutrophils (data not shown). These results clearly indicate that the increase in brain MPO activity after transient MCAO reflects neutrophil infiltration into the brain. In addition, depletion of neutrophils markedly attenuated brain edema formation and infarct size. It was noteworthy that edema formation was completely inhibited by depletion of neutrophils in the ischemic penumbra. As to the mechanisms of depletion of in vivo neutrophils by RP3, there is little information. Sekiya et al reported that peritoneal macrophages that showed phagocytosis of neutrophils were observed after administration of RP3. Phagocytosis of antibody-coated neutrophils by macrophages may be a candidate in the mechanism of neutrophil depletion. In our present study, we administered saline as the vehicle. We should have used nonimmune serum as control to remove the possibility that neutrophil depletion by antibody is not the cause of the neuroprotective effect of the antibody. Nevertheless, these results are strongly indicative of a contribution of neutrophils to brain injury under ischemia/reperfusion conditions. However, there is a possibility that depletion of neutrophils alters hemorheological factors that may contribute to the mechanism of protection. Further work is required to clarify the effect of neutrophil depletion on blood viscosity.
The roles of neutrophils in the pathogenesis of cerebral ischemia and stroke include the no-reflow phenomenon caused by vessel plugging and release of vasoconstrictive mediators, enhancement of vascular permeability, parenchymal injury by hydrolytic enzyme release, lipid mediator synthesis, and active oxygen species production. We assume that the depletion of neutrophils results in the reduced release of chemical mediators in the ischemic area and lightens the no-reflow phenomenon, thus suppressing postischemic brain injury.

Increased adherence of circulating neutrophils to the vascular endothelium is an essential early event in the initiation of an inflammatory response after a period of ischemia-reperfusion. However, the precise mechanism of the neutrophil adherence under the ischemia-reperfusion condition is still poorly understood. Recent reports have indicated that ischemia-reperfusion treatment increases CD11/CD18-ICAM-1-dependent neutrophil adherence to endothelial cells and that platelet-activating factor (PAF) and PAF-induced active oxygen species (H2O2 and superoxide anion) are responsible for this hyperadhesiveness. PAF is synthesized in the brain during ischemia or convulsions, and beneficial effects of PAF receptor antagonists have been demonstrated in various models of cerebral ischemia. Ferrichius et al reported that a PAF receptor antagonist attenuated neutrophil infiltration into the brain of rats with traumatic brain injury. We also observed that administration of a PAF receptor antagonist significantly reduced neutrophil infiltration into the ischemic penumbra after reperfusion after 1 hour of MCA occlusion (Y.M., unpublished data, 1993). Yoshida et al reported that a PAF receptor antagonist inhibits the adhesion-promoting effects of supernatants from endothelial cell exposed to 30 minutes of anoxia followed by 30 minutes of reoxygenation. Recently, evidence of an interaction between CD11/CD18 and ICAM-1, and H2O2 induction by PAF after ischemia, has been reported.

Compared to normoxic neutrophils, CD11/CD18-ICAM-1-dependent neutrophil accumulation in ischemic reperfused liver. PAF can directly stimulate the production of O2 and H2O2 in neutrophils, and active oxygen species produced in neutrophils by PAF may be involved in neutrophil accumulation in the ischemic tissue.

In conclusion, we have demonstrated that the increase in brain MPO activity after focal ischemia correlates well with the histologically identified infiltration of neutrophils and postischemic brain injury. The depletion of circulating neutrophils not only inhibited the increase of MPO activity in the ischemic brain but also attenuated postischemic brain injury. These results indicate that enhanced MPO activity after ischemia was derived from the infiltration of neutrophils into the ischemic brain and that this infiltration is implicated in postischemic brain injury. MPO activity is an excellent marker for the estimation of infiltrating neutrophils and brain injury after ischemia and reperfusion.

Acknowledgments

We thank Dr F. Sendo for donating RP3 and M. Okada and Y. Yamasaki for their valuable help.

References

The article by Matsuo et al is an important milestone in the elucidation of the role of inflammation in ischemic brain injury. Although the accumulation of leukocytes into ischemic (permanent or transient) brain has been repeatedly demonstrated, the significance of leukocyte accumulation on the final outcome of stroke (histologically and functionally) is far from being settled. The generation of the RP3 anti-rat neutrophil antibody has been extremely valuable in studying this issue because it provides a specific approach to isolate a particular circulating leukocyte and focus attention on its role in ischemic brain injury. The successful demonstration by various complementary methods that the prevention of neutrophil accumulation in the brain following ischemic injury is associated with lesser ischemia-induced histological and neurochemical deficits provides evidence in support of the possibility that cellular elements exogenous to the brain, in particular neutrophils, are important contributors to the injury process. In this respect, the study of Matsuo et al supports an important therapeutic opportunity to evaluate the therapeutic opportunities available in a clinical time frame (ie, less than 2 hours after ischemia), because depletion of leukocytes in a preventive mode (either by an RP3-like strategy or adhesion molecule antagonists) carries liabilities such as host-defense impairment and is therefore unrealistic for clinical use. Third, the mediators generated by the leukocytes that contribute to brain injury should be better understood. In this respect, one must keep in mind that reciprocal interactions between the leukocytes and brain cells (glia and neurons) may result in modified neurochemical profiles of either or both cells such that factors released from leukocytes may stimulate glia or neurons to release toxic factors (eg, cytokine) that then amplify the inflammatory process and aggravate the distressed environment.

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Stroke. 1994;25:1469-1475
doi: 10.1161/01.STR.25.7.1469

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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http://stroke.ahajournals.org/content/25/7/1469

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