In Vivo Evaluation of Leukocyte Dynamics in Retinal Ischemia Reperfusion Injury

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PURPOSE. To evaluate quantitatively leukocyte dynamics in vivo in the rat retinal microcirculation during ischemia reperfusion injury with the use of acridine orange digital fluorography.

METHODS. Retinal ischemia was induced in anesthetized pigmented rats by a temporary ligation of the optic nerve. After 60 minutes of ischemia, leukocyte behavior in the retinal microcirculation was evaluated, with acridine orange digital fluorography—consisting of a scanning laser ophthalmoscope and the fluorescent nuclear dye, acridine orange—during reperfusion at 1, 2, 4, 6, 12, 24, 48, 96, and 168 hours. The obtained images were recorded on videotape and analyzed with a computer-assisted image analysis system.

RESULTS. Rolling leukocytes along the major retinal veins were observed in treated rats during the reperfusion period; no rolling leukocytes were observed in the control rats. The number of rolling leukocytes gradually increased and peaked at 102 ± 40 cells/minute 12 hours after reperfusion; few rolling leukocytes were observed at 96 hours. The velocity of rolling leukocytes at 12 hours (19.1 ± 3.5 μm/second; P < 0.05) was significantly lower than that at the other three times. No rolling leukocytes were observed along the arterial walls throughout the experiments. The number of accumulated leukocytes increased as time elapsed, peaked at 931 ± 187 cells/mm² 24 hours after reperfusion, and decreased thereafter.

CONCLUSIONS. Leukocyte dynamics in the retinal microcirculation can be quantitatively evaluated during ischemia reperfusion injury. (Invest Ophthalmol Vis Sci. 1998;39:793–800)

Ischemia reperfusion injury has been reported in a variety of clinical and experimental conditions, including myocardial infarction; stroke; and mesenteric, renal, and peripheral vascular disease. In findings in many studies, leukocytes have been shown to accumulate in tissue after ischemia and to cause tissue injury by blocking blood flow or by producing the superoxide anion radical, hydrogen peroxide. In results of early experimental studies, it has been shown that leukocyte depletion greatly attenuates postischemic cellular dysfunction in various organs. Results in subsequent studies have demonstrated that the extent of reperfusion-induced tissue damage is reduced by agents that prevent leukocyte activation or accumulation. Therefore, it is suggested that leukocytes play a central role in ischemia reperfusion injury. The recruitment of circulating leukocytes into tissue after ischemia initially requires interaction between microvascular endothelial cells and these leukocytes through specific adhesion molecules. Recently, advances have been made in the understanding of the mechanisms of leukocyte adhesion and migration.

Because the retina belongs to the central nervous system and is vulnerable to ischemia, retinal ischemia reperfusion injury has been studied by many investigators. Although leukocytes may play an important role in ischemia reperfusion injury in the retina, leukocyte behavior in the retina after ischemia has been reported in a only few studies.

Acridine orange digital fluorography, which we developed recently, allows visualization of leukocytes and quantitative evaluation of leukocyte dynamics in the retinal microcirculation in vivo. In this technique, a scanning laser ophthalmoscope, coupled with a computer-assisted image analysis system, makes continuous high-resolution images of leukocytes stained by a metachromatic fluorochrome of acridine orange. The purpose of this study was to use acridine orange digital fluorography to evaluate leukocyte dynamics quantitatively in retinal ischemia reperfusion injury in vivo.

METHODS

Animal Model

Transient retinal ischemia was induced according to the method described by Stefansson et al., with slight modification. Male pigmented Long Evans rats (200–250 g; n = 65) were anesthetized with a mixture (1:1) of 4 mg/kg xylazine hydrochloride and 10 mg/kg ketamine hydrochloride. The pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. After a lateral conjunctival peritomy and...
disinsertion of the lateral rectus muscle, the optic nerve of the right eye was exposed by blunt dissection. A 6-0 nylon suture was passed around the optic nerve and was tightened until blood flow ceased in all of the retinal vessels. Complete non-perfusion was confirmed through an operating microscope and maintained for 60 minutes. Thereafter, nonperfusion was confirmed through an operating microscope, and the suture was removed. Reperfusion of the vessels was observed through the operating microscope. Eyes that failed to reperfuse within 5 minutes were excluded from the experiment. Rats in the sham-operation group underwent similar surgery but without tightening of the suture. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Acridine Orange Digital Fluorography**

Acridine orange digital fluorography has been described elsewhere. In this technique, a scanning laser ophthalmoscope (Rodenstock Instruments, Munich, Germany) coupled with a computer-assisted image analysis system makes continuous high-resolution images of fundus stained by the metachromatic fluorochrome, acridine orange (Wako Pure Chemicals, Osaka, Japan). Acridine orange is widely used in biochemical and cytochemical studies. The dye emits a green fluorescence when it interacts with double-stranded DNA. The spectral properties of acridine orange-DNA complexes are similar to those of sodium fluorescein, with an excitation maximum at 502 nm and an emission maximum at 522 nm. The argon blue laser was used for the illumination source, with a regular emission filter normally used in fluorescein angiography. Immediately after acridine orange solution was infused intravenously, leukocytes were stained selectively among circulating blood cells. Nuclei of vascular endothelial cells also were stained. The obtained images were recorded on an S-VHS videotape at the video rate of 30 frames/second for further analysis.

**Experimental Design**

Acridine orange digital fluorography was performed 1, 2, 4, 6, 12, 24, 48, 96, and 168 hours after reperfusion. In the sham-operation group, the examination was performed 4, 12, and 24 hours after surgery. Rats that did not undergo surgery were evaluated as a control. Five rats were examined at each time point after surgery. In preliminary experiments, arterial blood pressure was monitored by the blood pressure analyzer (model 179, IITC, Woodland Hill, CA). Mean arterial blood pressures...
were 93 ± 15 mm Hg in the control group, and 86 ± 14 mm Hg and 102 ± 10 mm Hg at 12 and 24 hours, respectively, after reperfusion in the operation group. No depression of arterial blood pressure was recognized.

Immediately before acridine orange digital fluorography, rats were anesthetized with the same agent, and the pupils were dilated. A contact lens was used to retain corneal clarity throughout the experiment. Each rat had a catheter inserted into the tail vein and was placed on a stereotaxic platform. Acridine orange (0.1% solution in saline) was injected continuously through the catheter for 1 minute at a rate of 1 ml/minute. The fundus was observed with the scanning laser ophthalmoscope in the 40° field for 5 minutes. Thirty minutes after the injection of acridine orange, the fundus was observed again to evaluate leukocytes accumulated in the retinal microcirculation.

After the experiment, the rat was killed with an anesthetic overdose, and the eye was enucleated to determine a calibration factor with which to convert values measured on a computer monitor (in pixels) into real values (in micrometers). The calibration factor is the ratio between the actual size of each optic disc measured using a microscope and the apparent value on a computer monitor.

**Image Analysis**

The video recordings were analyzed with an image analysis system, which has been described in detail elsewhere. In brief, the system consists of a computer equipped with a video digitizer (Radius, San Jose, CA), which digitizes the video image in real time (30 frames/second) to 640 horizontal and 480 vertical pixels, with an intensity resolution of 256 steps. To investigate the leukocyte dynamics in the microcirculation of the retina after ischemia, the investigators evaluated diameters of major retinal vessels, the flux of rolling leukocytes along the major retinal veins, the velocity of rolling leukocytes, and the number of leukocytes accumulated in the retinal microcirculation through the use of this system.

Diameters of major retinal vessels were measured at 1 disc diameter from the center of the optic disc in monochromatic images recorded before acridine orange injection. Each vessel diameter was calculated in pixels as the distance between the half-height points determined separately on each side of the density profile of the vessel image. The obtained data were converted into real values by using the calibration factor mentioned above. The averages of the individual arterial and venous diameters were used as the arterial and venous diameters for each rat.

Rolling leukocytes were defined as leukocytes that move at a lower velocity than free-flowing leukocytes. The number of rolling leukocytes was calculated from the number of cells crossing a fixed area of the vessel at a distance 1 disc diameter from the optic disc center per minute. The flux of rolling leukocytes was defined as the total number of rolling leukocytes along all major veins (cells per minute). The velocity of rolling leukocytes was calculated as the time required for a leukocyte to travel a given distance (25 pixels) along the vessel. The average of at least 10 velocities was defined as the velocity of rolling leukocytes for each rat.

The number of leukocytes accumulated in the retinal microcirculation was evaluated 30 minutes after acridine orange injection. The number of fluorescent dots in the retina within 8 to 10 areas of 100 pixels square at a distance of 1 disc diameter from the edge of the optic disc was counted. The average number of individual areas was used as the number of leukocytes accumulated in the retinal microcirculation in each rat.

**Statistical Analysis**

All values are presented as mean ± standard deviation. Analysis of variance was used to compare three or more conditions, with posthoc comparisons tested using Fisher’s protected least significant difference (PLSD) test. Differences were considered statistically significant when P values were less than 0.05.

**RESULTS**

**Diameters of Major Retinal Vessels**

In Figures 1 and 2, the time course of major retinal vessel diameters is shown in the nonsurgical control, sham-operation, and operation groups at various time points after reperfusion. In arteries and veins, vasoconstriction occurred immediately after reperfusion and peaked 4 hours after reperfusion in the operation group (66.8%; P < 0.01, 90.1%; P < 0.05). Afterward, significant vasodilation occurred in arteries and veins. In arteries, vasodilation peaked 12 to 24 hours after reperfusion (123-129%, P < 0.01) compared with the value at 4 hours after reperfusion and subsided 96 hours after reperfusion. Vasodilation was more remarkable in veins than in arteries. Venous vasodilation, which reached a peak 24 hours after reperfusion,
FIGURE 3. A leukocyte observed rolling along the venous wall (arrowhead). Another leukocyte rolled along the wall of a major retinal vein (closed arrow) and adhered to the venous wall (open arrow).

was more significant \( P < 0.05 \) in the surgical group than in the sham-operation group, and subsided 48 hours after reperfusion.

Rolling of Leukocytes

Immediately after acridine orange was infused, many free-flowing leukocytes were visualized. Because free-flowing leukocytes in the major retinal vessel move rapidly, it is likely that the image of a free-flowing leukocyte was nonspherical—that is, slightly elongated. It could be recognized as a single particle, however, even if the image was blurred. In some postischemic conditions, leukocytes were observed rolling along the major retinal veins, making intermittent adhesive contact with vascular endothelial cells. But no rolling leukocytes were observed in the major retinal arteries throughout all of the experiments (Fig. 3).

No rolling leukocytes were seen in the major retinal veins in the control, sham-operation, or operation group 1 hour after reperfusion; leukocytes began to roll along the venous walls 4 hours after reperfusion. The flux of rolling leukocytes substantially increased and reached a peak \( (102 \pm 40 \text{ cells/minute}) \) 12 hours after reperfusion. The flux decreased notably to approximately one sixth the maximum level 48 hours after reperfusion. Few rolling leukocytes could be observed 96 and 168 hours after reperfusion (Fig. 4).

Velocity of Rolling Leukocytes

The velocities of rolling leukocytes 2, 48, and 168 hours after reperfusion could not be determined, because insufficiently low numbers (fewer than 10) of rolling leukocytes were observed during the experiments. Leukocyte rolling velocities in the operation group 4, 6, and 24 hours after reperfusion were \( 28.5 \pm 3.7 \mu \text{m/second}, 26.5 \pm 3.4 \mu \text{m/second}, \) and \( 27.2 \pm 3.5 \mu \text{m/second} \), respectively, and there was no significant difference among them. The velocity of rolling leukocytes 12 hours after reperfusion, however, was significantly lower \( (19.1 \pm 3.5 \mu \text{m/second}; P < 0.05) \) than that recorded at 4, 6, and 24 hours (Fig. 5).

Leukocytes Accumulated in the Retinal Microcirculation

Because acridine orange is membrane permeable, it easily infiltrates through the vessel wall and diffuses into the retina. A few minutes after acridine orange injection was stopped, circulating leukocytes showed less fluorescence, probably owing to the washout effect. In contrast, leukocytes that accumulated in the retina remained fluorescent for approximately 2 hours. Therefore, leukocytes that accumulated in the retina were recognized as distinct fluorescent dots 30 minutes after acridine orange injection, although no circulating leukocytes fluor esced (Fig. 6).

In Figure 7 the changes in number of leukocytes accumulated in the retinal microcirculation are indicated. Although few leukocytes could be recognized in the control and sham-operation groups, accumulated leukocytes began to increase in the operation group 4 hours after reperfusion. The number increased with time and peaked at \( 931 \pm 187 \text{ cells/mm}^2 \) 24 hours after reperfusion. The number of accumulated leukocytes decreased after that and remained at approximately one third the maximum level.

DISCUSSION

In this study, leukocytes were clearly visualized and quantitatively evaluated rolling along the retinal vessel wall and accumulating in the retinal microcirculation after transient retinal ischemia in vivo. The rolling of leukocytes is the phenomenon in which leukocytes make intermittent adhesive contact with vascular endothelial cells\(^ 28 \) and is suggested to be the first step in the accumulation of leukocytes in tissue after ischemia\(^ 5,10,17 \). In the physiologic state, although rolling leukocytes are observed along the venous wall in some organs\(^ 16,29 \), no rolling leukocytes have been observed in the retinal major veins. Baatz et al.\(^ 30 \) have reported that the velocity of free-flowing leukocytes was \( 2.57 \pm 0.07 \text{ mm/second} \) in iris venules; Nishiwaki et al.\(^ 22 \) reported that the velocity was \( 17.4 \pm 5.3 \text{ mm/second} \) in the major retinal veins. From these reports, the velocity of free-flowing leukocytes is much faster in the retina than in the iris. The higher speed of flow in retinal vessels, compared with that in other vessels, may be why rolling leukocytes are absent in the normal retina. In a pathologic state, however, such as that occurring after ischemia, rolling leukocytes were observed along the venous wall against the higher flow speed. In this study, the velocity of rolling leukocytes ranged from 7.0 to \( 59.2 \mu \text{m/second} \) (average, \( 25.4 \pm 5.3 \mu \text{m/second} \)).
Leukocyte Dynamics in Retinal Ischemia Reperfusion

140
120
100
80
60
40
20
control
4
12
24
1
2
4
6
12
24
48
96
168
sham-operation
operation
Reperfusion period (hours)

FIGURE 4. Time course of flux of rolling leukocytes after reperfusion. Values are mean ± SD.

μm/second). Even the fastest rolling leukocyte moved almost 300 times more slowly than the average of free-flowing leukocytes. Therefore, by observing the monitor, it was possible to see two groups of leukocytes with two apparently different velocities: free-flowing and rolling leukocytes. Because no leukocytes with intermediate velocity were observed, it was not difficult to distinguish rolling leukocytes from free-flowing leukocytes.

In Figure 4, the time course of the number of rolling leukocytes is outlined. In this study, rolling leukocytes were first observed along the venous walls 4 hours after reperfusion. The number of rolling leukocytes increased substantially and reached a peak at 12 hours; they decreased to control levels at 96 hours. The time course in this study was delayed, compared with that in other organs. It is reported that rolling leukocytes peak 30 minutes after reperfusion and almost subside at 24 hours in previous studies; Szabo et al.13 reported that leukocyte infiltration to the retina increased after 90 minutes of retinal ischemia and 24 hours of reperfusion; 60 minutes of ischemia and 24 hours of reperfusion did not lead to migration. Hangai et al.8 showed that neutrophil migration in the retina subject to ocular ischemia for 2 hours was never observed before 3 hours after reperfusion; it was readily apparent after 12 hours. Although their observation is partially compatible with our findings, they may have underestimated the number of leukocytes. Because they observed accumulated leukocytes on histologic sections, they could not evaluate all leukocytes accumulated in the retina after ischemia. Conversely, our allows quantitative evaluation of all leukocytes accumulated in the retina after ischemia.

According to recent advances in understanding the mechanisms of leukocyte adhesion and migration, leukocyte rolling is thought to be mediated by interaction between the selectin family (P, E, and L-selectin) and its counter ligand, sialyl LewisX.28 Okada et al.33 showed significant and persistent upregulation of P-selectin after focal brain ischemia and reperfusion. Recently, Zhang et al.34 have shown that mRNA expression of E-selectin was upregulated in the cerebrum after transient middle cerebral artery occlusion and peaked 10 hours after reperfusion. We speculate that upregulation occurred in the retina after ischemia and that upregulated selectins mediated leukocyte rolling along the major retinal veins to accumulate in the retina after ischemia.

In this study, the velocity of rolling leukocytes 12 hours after reperfusion was significantly lower (19.1 ± 3.5 μm/second; P < 0.05) than the velocities at other times. The velocity of free-flowing leukocytes was 13.5 ± 5.1 mm/second in the control rats and 10.2 ± 2.8 mm/second, 12.2 ± 4 mm/second, 10.4 ± 3 mm/second at 4, 12, and 24 hours after reperfusion in the operation group. The velocity of free-flowing leukocytes showed no significant changes during the experiment. The difference in kind and density of adhesion molecules involved in leukocyte rolling at each time point may account for this finding.10,28

Whereas leukocytes make weak adhesive contact with vascular endothelial cells, they are activated by interaction with endothelial cells and adhere firmly to them.9,10,28 In this study, accumulated leukocytes began to increase substantially 4 hours after reperfusion and reached a peak level of 931 ± 187 cells/mm² at 24 hours. In previous studies, Szabo et al.13 reported that leukocyte infiltration to the retina increased after 90 minutes of retinal ischemia and 24 hours of reperfusion; 60 minutes of ischemia and 24 hours of reperfusion did not lead to migration. Hangai et al.8 showed that neutrophil migration in the retina subject to ocular ischemia for 2 hours was never observed before 3 hours after reperfusion; it was readily apparent after 12 hours. Although their observation is partially compatible with our findings, they may have underestimated the number of leukocytes. Because they observed accumulated leukocytes on histologic sections, they could not evaluate all leukocytes accumulated in the retina after ischemia. Conversely, our allows quantitative evaluation of all leukocytes accumulated in the retina after ischemia.

35
30
25
20
15
10
5
0
4
6
12
24
Leukocyte rolling velocity (μm/sec)

FIGURE 5. Velocities of rolling leukocytes at time points after reperfusion. The leukocytes' rolling velocity at 12 hours was significantly lower than values at 4, 6, and 24 hours. Values are mean ± SD. *P < 0.05 compared with the values at 12 hours.
FIGURE 6. Leukocytes accumulated in the retinal microcirculation appear as fluorescent dots 30 minutes after acridine orange injection. A small number of leukocytes could be seen in the control rats (A). Increasing numbers of leukocytes accumulated after reperfusion and peaked 24 hours after reperfusion in the surgical rats (B).

The β2-integrin family (CD11a/CD18, CD11b/CD18) and the immunoglobulin superfamily (intercellular adhesion molecule 1 [ICAM-1]), have been suggested as mediators in firm adhesion between leukocytes and endothelial cells.3,9,10,35 Although ICAM-1 is constitutively expressed on endothelial cells,15 Wang et al.35 have shown that mRNA expression of ICAM-1 is upregulated after transient cerebral ischemia and peaks 12 hours after reperfusion. Kim et al.36 have recently shown that CD11a and CD18 are upregulated in leukocytes from patients who have had ischemic stroke. Our observations in the retina are consistent with their results in the cerebrum, and we speculate that similar upregulation occurred in the retina after ischemia and that these upregulated adhesion molecules mediated the interaction between accumulated leukocytes and retinal vascular endothelial cells.

In this study, as is shown in Figure 1, significant vasoconstriction and subsequent vasodilation occurred in arteries and veins after reperfusion. Significant vasoconstriction was observed immediately after reperfusion and reached maximum levels 4 hours after reperfusion. Vascular endothelium is a source of relaxing (nitric oxide [NO]) and contracting factors (endothelin), and interactions between these factors contribute to the vascular tone.37,38 It is reported that endothelium injured by ischemia releases insufficient NO after reperfu-
This imbalance may contribute to marked vasoconstriction after reperfusion. On the contrary, Hangai et al. showed that mRNA expression of inducible nitric oxide synthase (iNOS) was highly upregulated 12 to 24 hours after reperfusion, when the retina was subjected to ischemia for 2 hours. They also showed that mRNA of iNOS was expressed in the resident retinal cells and neutrophils that had infiltrated the retina. As is shown in Figure 7, leukocytes began to accumulate in tissue after ischemia and peaked after 24 hours of reperfusion. A large amount of NO produced by accumulated leukocytes may contribute to significant vasodilation in arteries and veins after vasoconstriction. Hangai et al. also reported that mRNA expression of iNOS returned to normal levels 48 hours after reperfusion, a finding that is compatible with our results showing that vasoconstriction was dominant 48 hours after reperfusion. Accumulated leukocytes do not produce as much NO 48 hours after reperfusion.

In conclusion, rolling along the venous wall is essential for leukocytes to disseminate in tissue after ischemia and to produce tissue injury. Therefore, investigating leukocyte dynamics in the retina after ischemia is valuable in estimating ischemia reperfusion injury and in evaluating the protective effect of drugs. In the present study dynamic behavior of leukocytes was visualized in the retina after ischemia. The method used will allow quantification of the severity of ischemia reperfusion injury and quantitative evaluation of the effectiveness of therapies for retinal ischemia.

References


