Interactions Between Blood Cells and Retinal Endothelium in Endotoxic Sepsis

Akitaka Tsujikawa, Junichi Kiryu, Kenji Yamashiro, Atsushi Nonaka, Kazuaki Nishijima, Yoshihito Honda, Yuichiro Ogura

Abstract—Platelets and leukocytes are thought to play a leading role in the pathogenesis of many inflammatory conditions. To recruit flowing blood cells to the inflammatory region, it would be necessary for them to interact with vascular endothelial cells. Recently, many reports have indicated the resistance of spontaneous hypertensive rats (SHR) to endotoxic sepsis. Their resistance might be derived from suppressed interaction between these blood cells and endothelial cells. Therefore, SHR and age-matched Wistar-Kyoto rats (WKY) were induced with endotoxic sepsis by intravenous injection of lipopolysaccharide (LPS). At 4, 12, 24, and 48 hours after induction, leukocyte-endothelial interactions in the retina were evaluated in vivo with acridine orange digital fluorography. Fluorescently labeled platelets were also injected to investigate platelet-endothelial interactions in the retina in endotoxic sepsis. Leukocyte rolling in SHR after LPS injection was significantly suppressed; the maximum number of rolling leukocytes was reduced by 80.1% at 12 hours after LPS injection in SHR compared with WKY. Subsequent leukocyte infiltration into the vitreous cavity was significantly inhibited in SHR. Furthermore, platelet-endothelial interactions in the retina were also suppressed in SHR treated with LPS. The maximum numbers of rolling and adherent platelets were reduced by 59.5% and 62.6%, respectively, in SHR compared with WKY. In both strains, leukocyte- and platelet-endothelial interactions were substantially inhibited by the blocking of P-selectin. These suppressed interactions could contribute to the reduction of leukocyte- and platelet-mediated tissue injury in endotoxic sepsis in SHR, resulting in their resistance to endotoxemia. (Hypertension. 2000;36:250-258.)

Key Words: rats, inbred SHR ■ endotoxins ■ leukocytes ■ endothelium

Leukocytes are thought to play a leading role in the pathogenesis of many inflammatory conditions.1,2 Accumulated leukocytes have reportedly caused tissue damage by producing proteases and superoxide radical species and have accelerated the inflammatory process by producing various kinds of inflammatory cytokines. Moreover, growing evidence has suggested that platelets are also seriously involved in the pathogenesis of inflammatory tissue damage.3-5 Platelets could produce tissue damage through fibrin formation.6 On activation, accumulated platelets produce free radicals and proinflammatory mediators, such as serotonin, leukotrienes, thromboxane A2, and platelet-derived growth factor.5,7 Moreover, platelets reportedly have a potential to modulate the functional responses of leukocytes.8

Recent experiments investigating leukocyte adhesion to the vascular endothelium have shown that leukocyte accumulation in inflamed tissue is mediated through a multistep process, with each step mediated by distinct adhesion molecules.9,10 Similarly, to recruit flowing platelets to the inflammatory region, it would be necessary for platelets to interact with vascular endothelial cells.11 Growing evidence indicates that many kinds of adhesion molecules are expressed on platelets as well as vascular walls and that they function in concert to stabilize platelet adhesion under various flow conditions.12,13 Moreover, some intravital microscopic studies have reported that platelets can roll on activated endothelium through P-selectin, in the course of accumulation in the inflamed tissue.14,15

A recent in vivo study demonstrated that spontaneously hypertensive rats (SHR) showed resistance to septic shock.16 Many reports have indicated that SHR exhibit immune abnormalities with depressed leukocyte functions.17 Bernard et al16 have reported that lipopolysaccharide (LPS)-activated leukocytes from SHR produce significantly less tumor necrosis factor and interleukin-6. In addition, suppressed leukocyte involvement in inflammatory tissue injury in SHR is suggested to contribute to their resistance to endotoxin shock.18 Several intravital microscopic studies have shown suppressed leukocyte-endothelial interactions in SHR.18-21 However, little information is available about platelet-endothelial interactions in SHR under endotoxic sepsis.
The retinal microcirculation can be observed noninvasively, because of the optical media, which consist of the cornea, lens, vitreous body, and retina, are transparent. We have developed the technique of acridine orange (AO) digital fluorography, which allows us to evaluate leukocyte dynamics quantitatively in the retinal microcirculation in vivo. In this study, we have recently reported a method for the investigation of platelet-endothelial interactions noninvasively in the retinal microcirculation in vivo. With the use of this method, we have reported active platelet-endothelial cells in SHR under endotoxic sepsis.

The purpose of the present study was to evaluate quantitatively the interactions between leukocytes or platelets and retinal endothelial cells in SHR under endotoxic sepsis.

Methods

Animal Model

Twenty-week-old male SHR (n=77) and age-matched male nontensive Wistar-Kyoto rats (WKY, n=77) were used in the present study. All rats were fed standard chow ad libitum and were allowed free access to water in an air-conditioned room with a 12-hour light/12-hour dark cycle until they were prepared for the experiment. All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

LPS from Salmonella typhimurium (Difco Laboratories) was dissolved in sterile pyrogen-free 0.9% saline. LPS (2 mg/kg) was injected intravenously in rats of both strains to induce endotoxemia.

AO Digital Fluorography

AO digital fluorography has been previously described elsewhere. In this technique, a scanning laser ophthalmoscope (SLO, Rodenstock Instrument), coupled with a computer-assisted image analysis system, makes continuous high-resolution images of the fundus stained by the metachromatic fluorescein AO (Wako Pure Chemical). The dye emits a green fluorescence when it interacts with double-stranded nucleic acids (DNAs). The spectral properties of AO-DNA complexes are very 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 for fluorescein angiography. Immediately after the AO 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 for further analysis.

Evaluation of Leukocyte-Endothelial Interactions

Leukocyte-endothelial interactions in SHR and WKY were evaluated at 4, 12, 24, and 48 hours after LPS administration. Nontreated rats were evaluated as the control. Six different rats were used at each time point in each strain. Immediately before platelet administration, the rats were anesthetized with the same agent and prepared as described. Platelets (1×10⁸) were infused in the recipient rats of the same strain to measure the velocity of each platelet in the retinal vessels; thereafter, 2×10⁶ platelets were administered to evaluate the interactions with the retinal endothelial cells. The fundus was observed by SLO with the argon blue laser and a regular emission filter for fluorescein angiography. The obtained images were recorded on an S-VHS videotape for further analysis. In addition, fluorscently labeled platelets from WKY were administered to SHR 12 hours after LPS administration; platelets from SHR were also infused in WKY 12 hours after LPS administration (n=5).

P-Selectin Involvement in the Interactions

ARP-2, an anti-rat P-selectin monoclonal antibody (mAb), was a generous gift from Sumitomo Pharmaceuticals (Osaka, Japan). To evaluate the involvement of P-selectin in leukocyte- or platelet-endothelial interactions, we used SHR and WKY 12 hours after LPS injection. ARP-2 (2 mg/kg) was administered intravenously in each strain 5 minutes before the injection of AO solution or fluorscently labeled platelets (n=6 per each experiment).

Image Analysis

The video recordings were analyzed with an image-analysis system, which has been described in detail previously. In brief, the system consists of a computer equipped with a video digitizer (Radius). The latter digitizes the video image in real time (30 frames per second) to 640 horizontal and 480 vertical pixels, with an intensity resolution of 256 steps.

Diameters of major retinal vessels were measured at 1 disc diameter from the center of the optic disc in monochromatic images. 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 and converted into real values by using the calibration factor. 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 moved at a velocity slower than that of free-flowing leukocytes. The number of
rolling leukocytes crossing a fixed area of the vessel at a distance 1 disk diameter from the optic disc center was calculated per minute. The number of rolling leukocytes was defined as the total number counted along all major veins. The velocity of rolling leukocytes was calculated as the time required for leukocytes to travel a given distance along the vessel. The number of leukocytes that infiltrated the vitreous cavity was defined as the number of leukocytes in the vitreous cavity within a circle with a 1-disc-diameter radius from the center of the optic disc 20 minutes after AO injection.11

Rolling platelets were defined as platelets that moved at a slower velocity than free-flowing platelets in a given vessel. The number of rolling platelets was calculated from the number crossing a fixed area of the vessel at a distance 1 disc diameter from the optic disc center per minute. The number of rolling platelets was defined as the total number counted along all major veins. The velocity of rolling platelets was calculated as the time required for platelets to travel a given distance along the vessel. A platelet was defined as adherent to vascular endothelium if it remained stationary for >30 seconds. The number of platelets adherent to venous endothelial walls was quantified within a circle with a 500-μm radius from the center of the optic disc. All parameters were evaluated after a stabilization period of 5 minutes after the administration of platelets.11

To monitor the shear stress in each retinal vein, we substituted the maximal velocity (Vplt) of flowing platelets for the centerline red blood cell velocity. The mean red blood cell velocity (V) was estimated as Vplt/1.6. Calculation of the venous wall pseudoshear rate was based on the Newtonian definition: pseudoshear rate=(V/D)×8/π(=S) where D is the venular diameter.

After these experiments, the rats were killed with an overdose of anesthesia, and the eyes were enucleated to determine a calibration factor with which to convert values measured on a computer monitor (in pixels) into real values (in micrometers).

### Statistical Analysis

All values were presented as mean±SEM. The data were analyzed by ANOVA, with post hoc comparisons tested by the Fisher protected least significant difference procedure. Differences were considered statistically significant at P<0.05.

### Results

#### Diameters of Major Retinal Vessels

The Table indicates the physiological variables for both strains during the experiments. Leukocyte count in the peripheral blood decreased immediately after LPS injection in both strains but returned to beyond basal level thereafter. In contrast, the number of peripheral platelets, which was substantially reduced after LPS administration, remained low throughout the experiment. There were no significant differences in leukocyte and platelet counts between strains.

Figure 1 indicates changes of major retinal vessel diameters in control rats and at various time points after LPS administration. In arteries, slight vasodilation occurred gradually after LPS administration in both strains. Vasodilation reached 1.14-fold in WKY and 1.10-fold in SHR at its maximum compared with each control. In contrast, venous dilation after LPS injection was more substantial than was arterial dilation. Vasodilation reached 1.75-fold in WKY (P<0.0001) and 1.74-fold in SHR (P<0.0001) at its maximum compared with each control. In fact, diameters of major retinal arteries and veins of SHR were small at all time points compared with those of WKY (P=0.0062 and P<0.0001, respectively). However, no difference was found in the reactive vasodilation induced with LPS between SHR and WKY.

**Leukocyte-Endothelial Interactions**

Immediately after AO was infused intravenously, leukocytes were stained selectively among circulating blood cells. In LPS-treated rats, some leukocytes were observed slowly rolling along major retinal veins among many free-flowing leukocytes. None was seen along any major retinal arteries throughout the experiments (Figure 2A). No rolling leukocytes were observed along the major retinal veins in control rats in either strain. In WKY, a small number of leukocytes were observed rolling along the venous walls 4 hours after LPS injection. The number of rolling leukocytes substantially increased and peaked at 12 hours. In SHR, leukocyte rolling was significantly inhibited (P<0.0001, Figure 2B). The numbers of rolling leukocytes were reduced by 80.1% (P=0.0030) and by 85.3% (P=0.038) at 12 and 24 hours after LPS injection, respectively, in SHR compared with WKY. The velocity of rolling leukocytes at 12 hours after LPS injection in SHR and WKY was 44.2±2.4 and 36.0±1.9 μm/s, respectively. The velocity of rolling leukocytes in SHR was significantly faster than that in WKY (P=0.024).

Figure 3A shows the effect of P-selectin mAb ARP2-4 on the number of rolling leukocytes. In both strains, leukocyte rolling along the major retinal veins was substantially inhibited in ARP2-4–treated rats (P=0.0015); the numbers of rolling leukocytes at 12 hours after LPS administration were reduced by 53.7% and 75.1% (P=0.0002) in SHR and WKY, respectively, after ARP2-4 treatment.

AO easily infiltrates through vessel walls and diffuses into the retina because of the permeability of the membrane. Accordingly, a few minutes after AO injection was stopped, AO inflammation begins in the retina because of the permeability of the membrane.

### Table: Physiological Variables in Study and Control Rats

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<th>Parameters</th>
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<th>Control</th>
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<td></td>
<td>4 h</td>
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<td>24 h</td>
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<td>PLT, 10⁹/L</td>
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<td>Pseudoshear rate, 10⁹/s</td>
<td>2.74±0.11</td>
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Values are mean±SEM. WBC indicates peripheral leukocyte count; PLT, peripheral platelet count.

*P<0.01 and †P<0.05 vs WKY values at each time point; ‡P<0.01 and ¶P<0.05 vs control values in each group.
the fluorescence of circulating leukocytes was faint because of the washout. In contrast, leukocytes accumulated in the retina or infiltrating into the vitreous cavity remained fluorescent for ≈2 hours. Therefore, leukocytes that infiltrated into the vitreous cavity were recognized as fluorescent dots and were visible at 20 minutes after AO injection, although no circulating leukocytes fluoresced (Figure 2C). In both strains, few leukocytes could be found in the vitreous cavity until 24 hours after LPS injection. Thereafter, the number of leukocytes infiltrating into the vitreous cavity increased and peaked at 48 hours. The number of leukocytes infiltrating into the vitreous cavity significantly decreased in SHR compared with WKY ($P<0.0009$, Figure 2D). The number of leukocytes infiltrating into the vitreous cavity was reduced by 40.8% ($P=0.017$) 48 hours after LPS administration.

**Platelet-Endothelial Interactions**

Immediately after labeled platelets were infused intravenously, fluorescent platelets were visibly circulating in the retinal vessels. In LPS-treated rats, some platelets were observed slowly rolling along major retinal veins among many free-flowing platelets but not along any major retinal arteries throughout the experiments (Figure 4A and 4B). No rolling platelets were observed along the major retinal veins in the control in either strain. In WKY, some platelets were observed rolling along the venous walls 4 hours after LPS injection. The number of rolling leukocytes increased sub-

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<td>6.7±1.4</td>
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<td>329±87†</td>
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<td>1.47±0.07†</td>
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stantially and peaked at 12 hours. Rolling platelets were inhibited significantly in SHR compared with WKY ($P<0.0063$, Figure 4C). The maximum number of rolling platelets at 12 hours was significantly reduced by 59.5% ($P=0.036$) in SHR compared with WKY. The velocity of rolling platelets at 12 hours after LPS injection in SHR and WKY was 75.3±3.5 and 49.4±2.2 μm/s, respectively. The velocity of rolling platelets was significantly faster in SHR than in WKY ($P<0.0001$).

Changes in the numbers of platelets adhering to the retinal venous walls were seen in both strains (Figure 4D). Few adherent platelets were observed along the major retinal veins in the control in either strain. In WKY, platelets adherent to the venous walls increased after LPS injection and peaked at 12 hours. However, platelet adhesion was inhibited significantly in SHR compared with WKY ($P=0.0005$); the maximum number of adherent platelets at 12 hours was significantly reduced by 62.6% in SHR compared with WKY ($P=0.0079$). Furthermore, even if platelets harvested from WKY were injected into SHR at 12 hours after LPS administration, SHR did not show more active interactions between platelets and retinal endothelial cells. Platelets from SHR did have active interactions with endothelial cells in WKY (Figure 5).

In both strains, platelet rolling and adhesion along the major retinal veins were substantially inhibited in ARP2-4–treated rats ($P=0.0003$ and $P=0.0010$, respectively; Figure 3B and 3C). In ARP2-4–treated WKY, the numbers of rolling and adherent platelets 12 hours after LPS administration were reduced by 89.0% ($P=0.0005$) and 63.0% ($P=0.0006$), respectively. As a consequence of P-selectin inhibition, no significant differences could be observed in the number of rolling and adherent platelets between the strains.
Discussion

The recruitment of circulating blood cells into inflamed tissue initially requires interaction between microvascular endothelial cells and blood cells. In the present study, we investigated interactions between leukocytes or platelets and retinal endothelial cells in SHR and their normotensive counterpart, WKY, during endotoxic sepsis. In SHR, leukocyte rolling along the retinal major veins and subsequent leukocyte infiltration into the vitreous cavity were substantially inhibited. In addition, SHR showed significantly suppressed platelet rolling and adhesion along the major retinal veins throughout the experiments.

The importance of leukocytes in the pathogenesis of many inflammatory conditions has been supported by experimental studies to reduce inflammatory tissue damage by preventing leukocyte participation. Leukocyte adhesion to the endothelium has been shown to be mediated through a multistep process. The rolling phenomenon is the initial step and is prerequisite to firm adhesion and emigration at the sites of inflammation. In the mesentery, it has been reported that leukocyte rolling induced with LPS begins within 15 minutes and reaches its peak by 150 minutes. In the present study, however, leukocyte rolling was gradually upregulated after LPS injection and peaked at 12 hours. Leukocyte-endothelial interactions in the LPS-simulated retina occurred in a later phase than expected in other organs. There might be regional differences in leukocyte recruitment timing during endotoxic sepsis. It would appear from the present and previous studies that P-selectin plays a central role in leukocyte rolling in the LPS-stimulated retina. Our observations were supported by recent in vivo studies involving the expression of adhesion molecules. Miyamoto et al reported that mRNA expression of P-selectin was upregulated in the retina at 6 to 24 hours after LPS injection.

Moreover, accumulating evidence has suggested that platelets are also involved in the pathogenesis of inflammatory conditions, such as ischemia/reperfusion injury and endotoxic sepsis. Recently, Shibayama et al have reported a close relationship between the decrease of peripheral platelets and hepatocellular necrosis in endotoxic sepsis. In addition, Piguet et al have reported that platelet depletion with antiplatelet antibodies successfully affords protection against LPS-induced endotoxemia. Platelets can cause tissue damage not only through thrombus formation, but activated platelets also can produce free radicals and proinflammatory mediators. In addition, platelets are thought to potentially contribute to recruit leukocytes to the inflammatory region and to modulate leukocyte functional response.

Platelets normally circulate without firmly attaching to intact vascular endothelium, as shown in the control rats in the present study. Perhaps NO or prostaglandin I derived from endothelial cells might partially contribute to the antiplatelet property of the endothelium. Under low shear stress, rolling of activated platelets on high endothelial venules was reported, depending primarily on platelet P-selectin. Under higher shear stress, however, we and others have shown that even activated platelets reveal minimal interactions with unstimulated endothelial cells in the retina. In the present study, the LPS-stimulated retina exhibited active platelet-endothelial interactions in major retinal veins, which were substantially attenuated with the treatment of P-selectin mAb. Although P-selectin could be expressed on activated platelets, we have previously reported that the activation of platelets had a minor influence on platelet-endothelial interactions in the retina. Therefore, P-selectin on activated endothelial cells and not on platelets would mainly mediate platelet rolling. Our findings could be supported by intravital microscopic studies involving the
Mesentery of P-selectin–deficient mice. Those studies have shown that platelets from P-selectin–deficient and wild-type mice can roll along the stimulated endothelium of wild-type mice but not along the stimulated endothelium of P-selectin–deficient mice.

In the present study, leukocyte- and platelet-endothelial interactions in the retina during endotoxic sepsis were significantly attenuated in SHR. Many investigators have reported that SHR are more resistant to endotoxic shock than are WKY. Their resistance to endotoxic shock might be derived from the suppression of their interactions after LPS administration and after leukocyte- and platelet-mediated tissue damage. In the present study, moreover, treatment of P-selectin mAb attenuated leukocyte- and platelet-endothelial interactions in both strains to nearly the same level. Therefore, suppressed P-selectin–mediated interactions would

Figure 4. A, Fundus image of WKY 12 hours after LPS injection, which was administered with \(2 \times 10^6\) fluorescently labeled platelets. Many platelets were observed rolling or adhering along the major retinal veins among free-flowing platelets. However, it is difficult to discriminate rolling or adherent platelets from free-flowing platelets on a still picture. B, Monochromatic fundus image of WKY 12 hours after LPS injection (left). A time \(T\) sequence of 4 fluorescent fundus images of the same rat after administration of fluorescent platelets is shown to the right of the monochromatic image. Arrowheads indicate a rolling platelet along a major retinal vein; arrows indicate an adherent platelet. The second picture of the time sequence represents the image at 1 frame (0.033 seconds) after the first picture. The third and fourth pictures of the sequence represent the images at 30 and 300 frames (1 and 10 seconds, respectively) after the first picture (0 seconds). C and D, Time course of rolling (C) and adherent (D) platelets after LPS injection in both strains. Values are mean±SEM. *\(P<0.01\) and †\(P<0.05\) compared with values of control rats in each strain; ‡\(P<0.01\) and §\(P<0.05\) compared with values of WKY.
mainly contribute to the inhibition of blood cell recruitment to the retina in SHR during endotoxic sepsis. Moreover, considering the platelet behavior produced in rats of a different strain, the suppressed expression of P-selectin on the endothelial cells in SHR would account for our findings. Our findings support previous studies involving the expression of adhesion molecules in SHR.\textsuperscript{21} Suematsu et al\textsuperscript{20} have demonstrated suppressed P-selectin expression on endothelial cells in the histamine-stimulated mesentery of SHR. To date, the endogenous NO level\textsuperscript{18} and adrenal corticosteroid production\textsuperscript{19,21} have been suggested to be involved in suppressed interactions between leukocyte and endothelial cells in SHR. These agents might contribute to our findings in the retina under endotoxic sepsis, because several investigators have reported that endogenous NO and corticosteroid can suppress P-selectin expression.\textsuperscript{21,32}

In conclusion, P-selectin induced on retinal endothelial cells in endotoxic sepsis could contribute to interactions between the endothelium and leukocytes or platelets and be involved in the pathogenesis of inflammatory tissue damage.\textsuperscript{27,33} In the present study, SHR showed suppressed P-selectin-mediated interactions between retinal endothelial cells and leukocytes or platelets in LPS-induced endotoxic sepsis. This suppressed interaction might contribute to the resistance of SHR to endotoxic sepsis.

**Acknowledgment**

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**References**


Figure 5. Platelet behavior after platelet administration into rats of a different strain 12 hours after LPS administration. Platelet rolling (A) and platelet adhesion (B) are shown. Values are mean±SEM. \*P<0.01 and †P<0.05 compared with values in WKY administered with platelets from WKY.


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