In Vivo Evaluation of Platelet–Endothelial Interactions after Transient Retinal Ischemia

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PURPOSE. Accumulating evidence suggests that platelets play an important role in ischemia–reperfusion injury. To fulfill that role, platelets flowing in the bloodstream would have to interact with retinal endothelial cells and to accumulate in the postischemic retina. This study was designed to investigate quantitatively platelet–endothelial interactions in postischemic retina after transient retinal ischemia.

METHODS. Transient retinal ischemia was induced in Long-Evans rats for 60 minutes by temporal ligation of the optic nerve. Isolated platelet samples labeled with carboxyfluorescein diacetate succinimidyl ester were administered intravenously to recipient rats after various reperfusion periods. Platelet–endothelial interactions in postischemic retina were evaluated in vivo with a scanning laser ophthalmoscope. Anti-P-selectin monoclonal antibody (mAb) was administered 5 minutes before the injection of labeled platelets. P-selectin gene expression in the postischemic retina was studied by semi-quantitative polymerase chain reaction.

RESULTS. Under basal conditions, infused platelets showed minimal interactions with retinal endothelial cells. In contrast, postischemic retinas showed active platelet–endothelial interactions. Many platelets were observed rolling along and adhering to the major retinal veins. The number of rolling and adhering platelets reached a peak (555 ± 65/mm per min and 25.8 ± 3.2/mm2) 12 hours after reperfusion. However, the interactions between platelets and postischemic retinal endothelial cells were substantially inhibited by neutralizing P-selectin expressed on endothelial cells. In addition, P-selectin gene expression in postischemic retina corresponded with the time course of platelet–endothelial interactions during the reperfusion period.

CONCLUSIONS. This study demonstrated that platelets actively interacted with retinal endothelial cells in the postischemic retina through P-selectin expressed on the retinal endothelial cells. (Invest Ophthalmol Vis Sci. 2001;42:2102–2109)

A ccumulating evidence suggests that platelets play an important role in the pathogenesis of ischemia–reperfusion injury.1,2 The importance of platelets is supported by many previous studies that have demonstrated beneficial effects of platelet depletion against ischemia–reperfusion injury.3,4 Platelets undoubtedly contribute to thrombus formation, resulting in reocclusion of blood vessels.5 Moreover, activated platelets produce free radicals and proinflammatory mediators, such as serotonin, leukotrienes, thromboxane A2, monocyte chemotactic protein-3, and platelet-derived growth factor.6–10 In addition, platelets reportedly recruit leukocytes, which play a deleterious role in reperfusion injury, to ischemic regions through the expression of adhesion molecules on their surfaces or production of cytokines.11–15 Moreover, platelets can modulate leukocytes' functional responses.16,17

Under physiological conditions, platelets flowing in the bloodstream circulate without firm attachment to vascular endothelium. To recruit flowing platelets to the postischemic region, it would be necessary for platelets to interact with vascular endothelial cells through distinct adhesion molecules expressed on the surface of both the platelets and the cells. Recently, an intravitral microscopic study first reported that platelets can roll along endothelial cells in the postischemic mesentery in the course of accumulation during ischemia–reperfusion injury.18 P-selectin, the first adhesion molecule expressed on the postischemic endothelium, is thought to mediate these platelet–endothelial interactions.18 There is, however, little information about platelet–endothelial interactions in the retina during ischemia–reperfusion injury.

To study the role of platelets in postischemic retina, it is essential to investigate platelet behavior during the reperfusion period in vivo. Recently, we have developed a new in vivo method to quantitatively evaluate platelet–endothelial interactions in rat retina.19 With the use of this method, we have reported that activated platelets show minimal interactions with endothelial cells but that endothelial cells treated with lipopolysaccharide show active interactions with nonactivated platelets. This method could help to disclose the role of platelets in retinal ischemia–reperfusion injury. In this study, we evaluated platelet–endothelial interactions in vivo during reperfusion after transient retinal ischemia and investigated the molecular mechanism in these interactions.

MATERIALS AND METHODS

Animal and Ischemic Induction

Male pigmented Long-Evans rats (200–250 g) were used in this study. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Transient retinal ischemia was induced in the right eye of each rat, according to the method of Stefansson et al.,20 with slight modifications.21,22 Rats were anesthetized with a mixture (1:1) of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg). The pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. After lateral conjunctival peritomy and disinsertion of
the lateral rectus muscle, the optic nerve of the right eye was exposed by blunt dissection. A 60 nylon suture was passed around the optic nerve and tightened until blood flow ceased in all the retinal vessels. Complete nonperfusion was confirmed by visualization through a surgical microscope. After 60 minutes of ischemia, nonperfusion was confirmed through a surgical microscope, and the suture was removed. Reperfusion of the vessels also was observed through the surgical microscope. Control rats underwent similar surgery, but without tightening of the suture (sham operation).

Blood Sampling and Platelet Preparation
Carboxyfluorescein diacetate succinimidyl ester (CFDASE; Molecular Probes, Eugene, OR) is a nonfluorescent precursor that diffuses into cells and forms the stable fluorochrome carboxyfluorescein succinimidyl ester (peak absorbance, 492 nm; peak emission, 518 nm) after being catalyzed by esterase. This enzymatic reaction occurs predominantly in leukocytes and platelets and partially in serum. Intracellular fluorophores react with lysine residues of protein and remain within the cell as long as the membrane is intact.17

CFDASE was dissolved in dimethyl sulfoxide (Wako Pure Chemicals, Osaka, Japan) to a concentration of 15.6 mM, and a small aliquot (200 μl) was stored at −70°C until use. Platelet samples were prepared in accordance with the method described previously, with a slight modification.18 Blood samples from donor rats were harvested from the abdominal artery and collected in polypropylene tubes containing 0.136 M trisodium citrate, and 100 mM dextrose). The blood was centrifuged at the abdominal artery and collected in polypropylene tubes containing 2 mg/kg P-selectin monoclonal antibody (mAb, ARP2–4; Sumitomo Pharmaceuticals, Osaka, Japan) was administered to the recipient rats 5 minutes before they received labeled platelets (group 3, n = 6).25,26 Some platelet samples were preincubated with ARP2-4 at a concentration of 20 μg/ml while being labeled with CFDASE.27 These platelets were also infused into recipients that had gone 60 minutes of retinal ischemia at 12 or 24 hours after reperfusion (group 4, n = 6 at either time point).

Image Analysis
The video recordings were analyzed with an image-analysis system, consisting of a personal computer (Apple Computer, Cupertino, CA) equipped with a video digitizer (Radius, San Jose, CA). The latter digitizes the video image in real time (30 frames/sec) to 640 horizontal and 480 vertical pixels with an intensity resolution of 256 steps. We investigated the behavior of platelets in the retinal vessels to evaluate platelet–endothelial interactions.

Rolling platelets were defined as platelets that moved at a slower velocity than free-flowing platelets in a given vessel and that made intermittent adhesive contacts with vascular endothelial cells.18,28,29 The number of rolling platelets in each major retinal vein was calculated as the number of platelets rolling along each vein for 1 minute at a distance 200 μm from the optic disc center and the data expressed as platelets per venous diameter. The averages of the individual counts were used as the number of rolling platelets in each rat. Velocity of rolling platelets was calculated as the time required for a platelet to travel a given distance (30 μm) along the vessel. A platelet was defined as adherent to vascular endothelium if it remained stationary for longer than 10 seconds. Adherent platelets were calculated as the total number of adherent platelets along all major retinal veins for 1 minute within a circle with a radius of 500 μm from the center of the optic disc. The data are expressed as the number of adherent platelets per square millimeter of the endothelial surface of the major retinal veins. All parameters were evaluated after a stabilization period of 5 minutes after the administration of platelets.19

To monitor the venous wall shear rate in retinal veins, we substituted the maximal velocity of flowing platelets (Vmax) for the centerline red blood cell velocity. The mean red blood cell velocity (Vmean) was estimated as Vmax/1.6. The venous wall pseudoshear rate was calculated based on Poiseuille’s law for a Newtonian fluid: pseudoshear rate = (Vmean/D) × 8/3, where D is the venular diameter.30 Venular diameters were measured 200 μm from the optic disc center in monochromatic images recorded before the administration of platelets.

Semi-quantification of P-selectin Gene Expression in Postischemic Retina
The eyes were enucleated at 1, 2, 4, 6, 9, 12, 24, and 48 hours after reperfusion. Three rats were used at each time point. Each enucleated eye was cut into two pieces along the limbus, and then the retina was collected from the posterior segment. Nonischemic eyes were used as a control. Total RNA was isolated from the retina according to the acid guanidinium thiocyanate-phenol-chloroform extraction method.31 The extracted RNA was quantified, and then 5 μg of the RNA was used to make cDNA. cDNA was synthesized with a kit (First Strand; Pharmacia Biotech, Uppsala, Sweden). Polymerase chain reaction (PCR) was performed using the method of Saiki et al.32 and Nudel et al.,33 with slight modifications. The following conditions were used: denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, and polymerization at 72°C for 1 minute. The reaction was performed for 35 cycles for P-selectin and 25 cycles for β-actin. The primers were CAAGAGGAA-CAGCAAAGAAGACT (sense) and AATGGCTTCACAGGTTGGCA (antisense) for P-selectin and AGCTGAGAGAAGAATGTGGC (sense) and ACCAGAAGACTGTGGTG (antisense) for β-actin. Nucleotide sequencing and restriction pattern analysis confirmed that PCR products were derived from the target cDNA sequences.
Statistical Analysis
All values are presented as means ± SEM. The data were analyzed using a one-way analysis of variance using a post hoc test with the Fisher protected least-significance procedure. Differences were considered statistically significant at \( P < 0.05 \).

RESULTS

Platelet–Endothelial Interactions in Postischemic Retina
Table 1 indicates the physiological variables of the recipient rats. Whereas the pseudoshear rate in the major retinal veins decreased significantly after reperfusion, no significant differences were found in the pseudoshear rate through the time course of ischemia–reperfusion injury. The heart rate and blood pressure did not change significantly after the injection of labeled platelets.

Figure 1 shows the prepared fluorescent platelets. Immediately after labeled platelets were infused intravenously, they were visible as distinct fluorescent dots circulating in the retinal microcirculation. In the control or sham-operation rats, no platelets actively interacted with retinal endothelial cells. Even platelets harvested from rats with induced transient retinal ischemia showed minimal interaction with retinal endothelial cells. In the posts ischemic retina, however, some platelets were observed slowly rolling along major retinal veins among many free-flowing platelets, but not along any major retinal arteries (Fig. 2). Platelets began to roll along the venous walls at 4 hours after reperfusion. The number of rolling platelets substantially increased and reached a peak (555 ± 65/mm per min) at 12 hours after reperfusion and decreased almost to the basal level at 48 hours (Fig. 3). Platelets showed minimal interactions with arterial endothelial cells in the posts ischemic retina throughout the experiment.

The velocities of rolling platelets 1, 2, and 48 hours after reperfusion could not be determined, because insufficiently low numbers of rolling platelets were observed during those times. Figure 4 shows the velocity of rolling platelets along the major retinal veins after reperfusion. The velocity was substantially decreased 9 to 12 hours after reperfusion.

Most platelets rolling along the posts ischemic retinal veins were observed rolling away from the optic disc or flowing away downstream. Others had decreased in velocity and adhered to the vascular walls. Figure 5 indicates the number of platelets per square millimeter adherent to the venous wall after reperfusion. The number of adherent platelets substantially increased and reached a peak (25.8 ± 3.2/mm²) at 12 hours after reperfusion and decreased at 48 hours almost to the basal level.

Gene Expression of P-selectin in the Retina
Figure 6 indicates P-selectin mRNA expression in the retina after reperfusion. The levels of gene expression are shown as a ratio to the average expression in control rats. P-selectin gene expression gradually increased immediately after reperfusion and reached a maximum 9 to 24 hours after reperfusion.

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<th>Control</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>9</th>
<th>12</th>
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<td>117 ± 6</td>
<td>124 ± 8</td>
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<tr>
<td>Pseudoshear rate (10⁻³/sec)</td>
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<td>1.13 ± 0.16</td>
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**FIGURE 1.** Fluorescent micrograph shows prepared platelets stained with CFDASE. No aggregated platelets were recognized. Original magnification, ×400.
Effect of ARP2-4 on Platelet–Endothelial Interaction

In group 1, labeled platelets, which were harvested from normal rats, showed active interactions with retinal endothelial cells at 12 and 24 hours after reperfusion (Fig. 7). Even if infused with platelets harvested from donor rats with induced transient retinal ischemia and reperfusion, recipient rats did not show increased platelet–endothelial interactions in the retina (group 2). However, intravenous administration of P-

**Figure 2.** A sequence of fluorescent fundus images 12 hours after reperfusion was made after administration of fluorescent platelets. Platelets could be recognized as fluorescent dots in retinal microcirculation. Arrows: platelets adherent to venous endothelium; arrowhead: a platelet rolling along a major retinal vein. The other dots are free-flowing platelets. Their velocities were markedly different from those of rolling platelets as observed on a video monitor. T, number of seconds elapsed after administration of fluorescent platelets.

**Figure 3.** The number of platelets rolling along the major retinal veins after reperfusion was recorded. The number was calculated as the average number of platelets crossing an imaginary perpendicular line through main veins at a distance of 200 μm from the optic disc. Data are expressed as mean platelets per minute per venous diameter (±SEM). *P < 0.0001, †P < 0.01, compared with data in control rats. n = 6 for each group.

**Effect of ARP2-4 on Platelet–Endothelial Interaction**

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**Figure 4.** Velocity of rolling platelets along the major retinal veins was calculated after reperfusion. The platelets' rolling velocity at 9 hours was significantly lower than values at 6 and 24 hours. Data are means ± SEM. *P < 0.001 compared with the values at 24 hours and †P < 0.05 compared with the mean number of cells at 6 hours in control rats. n = 6 for each group.
selectin mAb to the recipient rats significantly attenuated platelet-endothelial interactions in the postischemic retina (group 3). The number of rolling platelets in the P-selectin mAb-treated rats was reduced by 50.3% ($P = 0.0074$) and 68.9% ($P = 0.034$) at 12 and 24 hours, respectively, compared with the number in group 1. The velocity of rolling platelets was significantly faster than in nontreated rats ($P = 0.0006$). In addition, P-selectin inhibition in the recipient rats reduced the number of adherent platelets by 57.4% ($P = 0.0064$) and 86.1% ($P = 0.0054$) at 12 and 24 hours, respectively. However, when P-selectin expressed on the platelets was blocked by preincubation with mAb, postischemic retina similarly showed active platelet-endothelial interactions (group 4).

**DISCUSSION**

In the present study, we first quantitatively evaluated platelet-endothelial interactions during retinal ischemia-reperfusion injury in vivo with the use of CFDASE. Whereas platelets in control and sham-operation rats showed minimal interactions with retinal endothelial cells, many platelets were observed rolling and adhering along the major retinal veins in postischemic retina. However, these interactions in the postischemic retina were substantially blocked by neutralizing P-selectin expressed on endothelial cells. P-selectin gene expression in the postischemic retina corresponded with the time course of platelet-endothelial interactions during the reperfusion period.

Accumulating evidence has suggested that platelets recruited in the postischemic tissue play a pivotal role in the pathogenesis of ischemic reperfusion injury.1,2 The importance of platelets is supported by many animal studies that...
have demonstrated beneficial effects of platelet depletion during ischemia–reperfusion injury. Platelets certainly contribute to thrombus formation, resulting in reocclusion of the blood vessels. Previous in vitro experimental studies have shown that accumulated platelets can generate free oxygen radicals and release various kinds of inflammatory cytokines. These cytokines recruit leukocytes to the ischemic region, which leads to postischemic tissue injury. Moreover, platelets can support leukocyte adherence to vascular endothelium and can modulate leukocyte functional response. Recently, Campbell et al. have reported that platelets and neutrophils act synergistically to provoke ischemia–reperfusion injury in the heart. In their report, hearts perfused with platelets and neutrophils exhibited remarkable cardiac dysfunction, compared with those perfused with only platelets or neutrophils. Platelets caused postischemic tissue damage not only by themselves but also in cooperation with leukocytes.

In the present study, platelets in the control rats circulated normally without firmly attaching to intact vascular endothelium. Nitric oxide or prostaglandin I2 derived from endothelial cells may partially contribute to the antiplatelet property of the endothelium. Under low shear stress, rolling of activated platelets on endothelial venules was reported, depending primarily on platelet P-selectin. However, we have shown that even activated platelets revealed minimal interactions with unstimulated endothelial cells in the retina due to higher shear stress. Figure 4 shows the time course of the number of rolling platelets in the postischemic retina. Rolling platelets were first observed along the venous walls 4 hours after reperfusion. The number of rolling platelets substantially increased and reached a peak at 12 hours, which is comparable to the peak of rolling leukocytes reported by Tsujikawa et al. It is possible that rolling platelets were attaching to leukocytes rolling along the postischemic venous walls; however, we think that most rolling platelets were not attached to rolling leukocytes, because the velocity of a rolling platelet in the present study was 71 to 120 μm/sec, or three to seven times faster than rolling leukocytes. Although some platelets observed rolling slowly and irregularly along the major retinal veins in the postischemic retina might have adhered to the rolling leukocytes, the ratio would be quite small.

It is well known that leukocyte adhesion to the endothelium is mediated through a multistep process. Rolling is the first step and a prerequisite for leukocytes to subsequently firmly adhere and migrate. The concept of platelet rolling on endothelial cells is relatively new and its significance is therefore still controversial. Figure 5 shows the time course of platelet adhesion along the postischemic retinal veins. The time course is parallel with that of platelet rolling. No platelets adhered along the retinal veins in control rats or in surgical subjects before 4 hours after reperfusion. The number of adherent platelets substantially increased and reached a peak at 12 hours. Some platelets rolling along the postischemic retinal veins were observed rolling away from the optic disc or flowing away downstream; others decreased in velocity and adhered to the vascular walls. All evidence taken together, rolling of platelets would be the initial stage of the adhesion of platelets to endothelial cells.

Recently, experiments investigating platelet–endothelial interactions by intravital microscopy have shown that platelet recruitment to the inflammatory region is mediated through specific adhesion molecules. P-selectin expressed on endothelial cells is thought to mediate these interactions. In the present study, intravenous administration of P-selectin mAb substantially attenuated platelet–endothelial interaction in the postischemic retina. Initially, P-selectin was thought to be expressed by rapid secretion from storage granules within a few minutes after stimulation with agents such as thrombin.
and to return to normal levels within a few hours. Subsequent studies, however, demonstrated that P-selectin synthesis and endothelial surface expression can be regulated by inflammatory cytokines. P-selectin expression in endothelial cell monolayers exposed to hypoxia and reoxygenation shows a biphasic response that initially peaks at 30 minutes, with a second peak in surface expression at 4 to 6 hours after reoxygenation. Figure 6 shows the time course of P-selectin gene expression in the postischemic retina. Expression gradually increased immediately after reperfusion and reached a maximum 9 to 24 hours after reperfusion. The time course of expression was delayed, compared with that in experiments in vitro. Similarly, Suzuki et al. showed that P-selectin immunoreactivity begins to be expressed in the microvascular vessels in the cerebral cortex at 2 hours after reperfusion and that the expression reaches a maximum at 8 hours to 1 day. P-selectin expression on retinal endothelial cells occurred for longer periods than initially expected.

Activated platelets express P-selectin on their surfaces by rapid secretion from α-granules. In the present study, although intravenous administration of P-selectin mAb showed active interactions with the postischemic retinal veins. Although some platelets harvested from donor rats may have been activated and may have expressed P-selectin on their surface, P-selectin expressed on the platelets would have little influence on platelet-endothelial interactions. Therefore, P-selectin would mainly mediate platelet rolling on activated endothelial cells and not on platelets. Our findings are supported by intravital microscopic studies with the mesentry of P-selectin−deficient mice reported by Massberg et al. They showed that platelets from P-selectin−deficient and wild-type mice can roll along postischemic endothelium in wild-type mice but not in P-selectin−deficient mice.

In the present study, platelets harvested from control rats showed similar interactions, with venous endothelial cells in the postischemic retina through P-selectin expressed on the endothelial cells, compared with those from rats with induced transient retinal ischemia (Fig. 7). Therefore, the ligand against P-selectin would mainly mediate platelet rolling on activated endothelial cells, which contribute to platelet–endothelial interactions in the postischemic retina.

In conclusion, we demonstrated that platelets, similarly to leukocytes, roll and firmly adhere along venous endothelial cells in the retina during ischemia–reperfusion injury. These interactions were mainly mediated by P-selectin expressed on the retinal endothelial cells. We have reported that blocking of P-selectin significantly attenuates retinal ischemia–reperfusion injury. This protective effect may be partially based on the inhibition of platelet–endothelial interactions in the postischemic retina.

References


