Rolling in P-Selectin–Deficient Mice Is Reduced But Not Eliminated in the Dorsal Skin

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P-selectin–mediated rolling is believed to be important in the recruitment of leukocytes to tissue after ischemia-reperfusion injury. The dorsal skin chamber was used to examine differences in the rolling and stable adhesion of circulating leukocytes in subcutaneous (SC) vessels of P-selectin–deficient and age-matched wild-type mice, both under basal conditions and after ischemia-reperfusion. Rolling in the postcapillary venules in SC tissue of P-selectin–deficient mice was significantly lower than that in wild-type mice under the basal conditions and post-ischemia-reperfusion (P < 0.06), but was not eliminated by the deletion of the P-selectin gene. No significant difference between P-selectin–deficient and wild-type mice in shear rate or leukocyte-endothelial adhesion was observed up to 24 hours after ischemia-reperfusion. These results show that P-selectin–mediated rolling is not a prerequisite for ischemia-reperfusion–induced leukocyte-endothelial adhesion in the skin.

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THE PARADIGM for leukocyte recruitment is rolling on the venular wall, followed by firm adhesion and extravasation. Families of leukocyte adhesion molecules have been implicated in this process. The selectins (P, L, and E) support the transient interaction of leukocytes with the endothelial wall, which translates to leukocyte rolling on the endothelium. The IgG superfamily members, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), on endothelial cells, with their cognate β2 and β1 integrin receptors on the leukocytes, mediate firm adhesion, although β1 integrins can also mediate rolling. Functional blocking monoclonal antibodies to leukocyte adhesion molecules significantly attenuate reperfusion injury, suggesting that, although other physiologic parameters such as hydrodynamic forces and products of activated neutrophils and endothelial cells are contributing factors, the adhesive interactions of leukocytes with the endothelium may be the rate-limiting steps.

The earliest-known adhesive interactions of leukocytes with the endothelium are mediated through the selectin family of adhesion molecules. All three selectins have been shown to mediate leukocyte rolling and leukocyte recruitment in vivo. P-selectin, constitutively present in the alpha granules of platelets and the endothelial cell–specific storage granules, Weibel–Palade bodies, is expressed on the cell surface minutes after activation of these two cell types with agonists such as thrombin and histamine. It mediates interaction with a variety of leukocytes, including neutrophils, lymphocytes, and monocytes. Within 20 minutes of surface expression, P-selectin is re-endocytosed and rerouted into the Weibel–Palade bodies. Ischemia leads to the production of oxygen-derived free radicals that promote reperfusion-induced leukocyte adherence and cause rapid P-selectin surface expression in vitro. Oxidants prolonged surface expression of P-selectin up to 4 hours, possibly by blocking the re-endocytosis of P-selectin. Tumor necrosis factor alpha (TNFα) and interleukin (IL)-1 were also recently shown to upregulate P-selectin with a time course similar to that of E-selectin expression, which is a strictly cytokine- and endotoxin-inducible endothelial selectin. L-selectin is constitutively present on a number of leukocyte subsets. Recent studies on animals genetically deficient in these selectins have shed light on the temporal sequence of selectin functions, as well as on their individual contributions in acute inflammatory models. Analysis of leukocyte rolling by intravital microscopy of mesenteric venules of P-selectin–deficient and L-selectin–deficient mice revealed an important role for P-selectin in leukocyte rolling soon after tissue insult, whereas L-selectin–mediated rolling appeared to be more prominent 1 to 2 hours after the onset of injury.

The relative roles of the selectins may depend on the particular mediators of the inflammatory model being studied. Antibodies against P-selectin reduced complement-induced lung injury, whereas antibodies against P-selectin attenuated IgG-immune complex–mediated lung injury. The heterogeneity in constitutive P-selectin expression in the different vascular beds suggests that its role in acute inflammation may also depend on the tissue in which the injury has occurred. In addition, because P-selectin is also present in platelets, the involvement of platelets in a given inflammatory model may accentuate the importance of P-selectin. P-selectin antibody has been shown previously to attenuate reperfusion injury to the rabbit ear and ischemia reperfusion injury to the feline heart. In skin, P-selectin is constitutively expressed in blood vessels in a punctate pattern that is typical of granule staining and has been implicated in both baseline leukocyte rolling and as the post–ischemia-reperfusion rolling increase through the use of monoclonal antibody blocking. The present study was designed to characterize the role of P-selectin in leukocyte-
endothelial interactions in the dorsal skin fold chamber implanted into P-selectin-deficient mice, both under basal conditions and after ischemia-reperfusion.

MATERIALS AND METHODS

Dorsal skin chamber implantation. Dorsal skin chambers were implanted into mice according to a previously described procedure,27 permitting observation of the vessels in the dorsal skin. P-selectin-deficient11 and age-matched wild-type control mice, weighing 25 to 35 g each, were implanted with chambers and held for a minimum of 3 days before use in an experiment.

Observation of rolling leukocytes in skin venules. Observations of blood flow were made without anesthesia. Chamber-bearing mice were placed into a polycarbonate holding tube to restrict mobility during observation; placed onto a polycarbonate viewing stage, and mounted on an intravital microscope (Axioplan; Zeiss, Oberkochen, Germany) equipped with a fluorescence filter set for rhodamine and fluorescein isothiocyanate (FITC) (Omega Optical, Brattleboro, VT), an intensified CCD video camera (C2400-88; Hamamatsu Photonics KK, Hamamatsu, Japan), a CCD video camera (AVC-D7; Sony, Tokyo, Japan) and a S-VHS videocassette recorder (SVO-9500MD; Sony, Tokyo, Japan).

Each chamber was initially mapped with the 10× and 20× objectives to select vessels of appropriate size and establish their location within the chamber relative to the other vessels. A minimum of three postcapillary venules in the 12- to 31-μm size range were selected for observation in each chamber. Each vessel was observed under transmitted light for at least 1 minute to obtain red blood cell (RBC) velocity data. An injection was then made with a bolus of 50 μL of 0.1% Rhodamine 6G (Molecular Probes, Eugene, OR) in saline into the tail vein to observe the leukocyte-endothelial interactions. Observations were made with the 20× objective using the intensified CCD camera and were limited to a 30-second period for each vessel.

Dorsal ischemia in the dorsal chamber. After the determination of baseline parameters, the dorsal skin was subjected to clamp hypoxia for 4 hours. This was performed with a screw clamp that was manufactured to fit over the rear of the skin window.22 As the clamp was gently closed, a plastic disk covered with thin rubber cushion compressed the skin against the window, thus restricting blood flow. The termination of flow was visually confirmed as the clamp was tightened, to avoid excess compression of the skin. After the ischemic period, the clamp was removed, and the chamber was monitored for blood flow and leukocyte-vascular interactions at 0.5, 3, 6, and 24 hours after reperfusion.

Analysis of data. The four-slit technique was used to establish the average RBC velocity in the dorsal skin chamber, with the recorded images obtained with transmitted light using the four-slit apparatus (MicroFlow System, model 208C, video photometer version; IPM, San Diego, CA) equipped with a personal computer (IBM PS/2, 40SX; Boca Raton, FL).

The diameter of the vessels was measured using an image-shearing device (Model 908, IPM). The shear rates of the observed vessels were calculated as: wall shear rate = 8 (average RBC velocity/ vessel diameter).

Leukocyte-endothelial interactions were quantified according to the method of Atherton and Born.24 The numbers of rolling (Nr) and adhering (Nn) leukocytes were counted for 30 seconds along a 200-μm section of vessel. The total flux of cells passing through this region during that period was also determined (Nt). The percent of rolling leukocytes was defined as 100 × Nr/(Nn + Nt). In a similar fashion, the density of adherent leukocytes (cells per square millimeter) was determined as [Nn × 104/(π × D × 200)], where D is the vessel diameter. The Mann-Whitney U test was used to test the statistical significance.

RESULTS

Baseline leukocyte rolling. The postcapillary venules of the dorsal skin showed a high level of baseline rolling. Approximately 68% of observed leukocytes were found to roll in the postcapillary venules in the dorsal skin of wild-type mice. In P-selectin-deficient mice, however, baseline rolling was reduced to 28% (P = .0015; Fig 1). Blood flow rates and vessel diameters were comparable between P-selectin-deficient and wild-type mice; consequently, there was no significant difference in the shear rates in vessels between the two groups (Fig 2).

Effects of ischemia-reperfusion on leukocyte-endothelial interactions. Wall shear rate, leukocyte rolling, and stable adhesion measured at 0.5, 3, 6, and 24 hours after reperfusion are shown in Figs 2 to 4. Microvascular diameters did not change throughout the experiment (data not shown). Also, RBC velocity and, therefore, wall shear rates did not significantly increase (Fig 2) in either the wild-type or P-selectin-deficient group when compared with the preischemic values, and no significant difference in wall shear rate could be detected between the two groups. At 0.5 hours postreperfusion, leukocyte rolling in wild-type mice was observed to be over 80% of the total leukocyte flux, but this apparent increase was not statistically significant when compared with the preischemia values (Fig 3). In parallel experiments, ischemia-reperfusion led to a similar profile of leukocyte rolling in P-selectin-deficient mice (Fig 3). However, the percent-
age of leukocytes rolling in P-selectin-deficient mice remained far below that seen in wild-type animals for all time points studied ($P < .05$).

There was a statistically significant increase in leukocyte adhesion after reperfusion in both the wild-type mice ($P < .05$, at time $t = 30$ minutes) and the P-selectin-deficient mice ($P < .05$, at $t = 6$ hours), and the number of adhering leukocytes returned to preischemic levels by 24 hours (Fig 4). On the other hand, leukocyte adhesion was comparable between wild-type and P-selectin-deficient animals (Fig 4).

**DISCUSSION**

We report a role for P-selectin in leukocyte rolling under baseline conditions and after ischemia-reperfusion in the skin microvasculature. Constitutive leukocyte rolling has been previously reported in the skin microvasculature of frog and hairless mice. In the study by Mayrovitz, it appears that leukocyte rolling occurs in homeostasis, because the leukocyte rolling was observed in the absence of surgical trauma. These rolling leukocytes may possibly be part of the surveillance mechanism in the skin microvasculature, because this tissue is constantly exposed to external insult. The adhesion molecules responsible for this rolling are not known. We now show that baseline rolling is a prominent feature in the dorsal skin window and that a deficiency in P-selectin leads to a dramatic decrease in the number of rolling leukocytes. As P-selectin is constitutively present in the skin microcirculation, it is plausible that P-selectin on the endothelium is responsible for supporting the baseline rolling in the skin. As the skin windows were in place for several days before intravital microscopy and were used only if no observable edema had developed during this period, the baseline rolling may represent the constitutive rolling pool of leukocytes in the dorsal skin and not the result of chronic tissue stimulation. Observations of rolling leukocytes in the skin of the ears of C3H and severe combined immunodeficiency (SCID) mice (our unpublished observations, January 1995) and hairless mice also show high
frequency rolling, suggesting that this may be a general property of skin vasculature. The presence of rolling leukocytes in the skin of P-selectin–deficient mice is unlike that seen previously in the mesenteric vasculature, where no leukocyte rolling was observed immediately after exteriorization of the mesentery. The differential role of P-selectin in these two tissues may reflect different levels of basal P-selectin expression or the expression of other adhesion molecules. However, the difference may also be due, in part, to the decreased shear rate present in the skin microvasculature as compared with the mesentery. At lower shear rates, other adhesion molecules may play a more prominent role.

Ischemia-reperfusion in the dorsal skin led to an insignificant increase in leukocyte rolling (P > .05), but a larger increase in leukocyte adhesion (P < .05), suggesting that the reperfusion-induced increase in leukocyte adhesion was not solely a consequence of an increase in P-selectin–mediated rolling. Instead, reperfusion appeared to have a dramatic effect on the number of leukocytes that successfully adhered, perhaps due to the release of inflammatory mediators that specifically effect firm adhesion of leukocytes. Ischemia-reperfusion is associated with decreased nitric oxide release, which leads to increased polymorphonuclear leukocyte adhesion. Platelet-activating factor (PAF), which plays an important role in mediating leukocyte adhesion after reperfusion of mesenteric venules, was shown in vivo to increase the number of adherent leukocytes without affecting the rolling pool. We have shown that after ischemia-reperfusion in the mouse skin, leukocyte adhesion and not leukocyte rolling is the primary parameter affected in both wild-type and P-selectin–deficient mice. Even though a deficiency in P-selectin has an effect on leukocyte rolling, it does not significantly attenuate reperfusion-induced leukocyte adherence in the skin. Our observations also indicate that rolling and adhesion are not inextricably linked, because lower levels of rolling, as seen in P-selectin–deficient mice, induced levels of adherent cells that were similar to those in wild-type animals. A similar lack of one-to-one correlation between rolling and firm adhesion has been also reported in thioglycollate-treated mesenteric preparation.

A possible explanation for this phenomenon may be that the levels of rolling observed is above a threshold value needed for the full attainment of firm adhesion during the reperfusion period.

It is possible that different leukocyte populations are interacting with the endothelium at different times. The problem of not being able to identify a subpopulation of leukocytes is inherent in experiments that use labeling of leukocytes in vivo. While we can observe the generalized outcome of the leukocyte interactions, we cannot determine which cell types are participating in this phenomenon. Hence, the observed interactions are likely to involve a diverse population of circulating lymphocytes, monocytes, and granulocytes.

Anti–P-selectin monoclonal antibody has been previously used to eliminate baseline rolling of leukocytes in the hairless mouse dorsal skin chamber. While our findings are qualitatively in agreement with these studies, we have found that deletion of P-selectin expression does not abolish leukocyte rolling, unlike the antibody-blocking studies. These differences may reflect (1) differences in basal expression of adhesion molecules between mice used in the different studies, (2) possible compensatory expression of other adhesion molecules, such as E-selectin or L-selectin ligands or VCAM, in the skin of the P-selectin–deficient mice, or (3) possible undetected crossreaction or other effects of the P-selectin monoclonal antibodies used in previous studies. Recent in vitro studies have demonstrated the participation of β1 integrins in the process of capture and rolling of lymphocytes under physiologic shear stress. As the shear rates are reduced in the skin compared with the mesentery, the integrins may be sufficient to support leukocyte rolling in the skin but not in the mesentery. In addition, basal expression of various adhesion receptors capable of inducing rolling may be different in vessels of the skin and connective tissue.

These studies have shown that rolling of leukocytes in the skin venules of P-selectin–deficient mice is reduced when compared with wild-type control mice, but not eliminated.
This suggests the contribution of other adhesion molecules in addition to P-selectin in promoting leukocyte rolling in this site. Furthermore, increased leukocyte-vascular adhesion after ischemia-reperfusion injury does not require an increase in P-selectin-mediated rolling in post-ischemia-reperfusion injury.

REFERENCES

29. Wagner R: Erlauterungstafeln zur Physiologie und Entwicklungs geschichte. Leipzig, Germany, Leopold Voss, 1839
36. Johnson RC, Mayadas TN, Frenette PS, Mebius RE, Subra-
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