Delayed Onset of Inflammation in Protease-Activated Receptor-2-Deficient Mice[1]

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Endothelial surface expression of P-selectin and subsequent leukocyte rolling in venules can be induced by mast cell-derived histamine and binding of thrombin to protease-activated receptor-1 (PAR1). We hypothesized that activation of endothelial PAR2 by mast cell tryptase or other proteases also contributes to inflammatory responses. Leukocyte rolling flux and rolling velocity were assessed by intravital microscopy of the cremaster muscles of wild-type mice following perivenular micropipette injections of a control (LSIGRL) or PAR2-activating (SLIGRL) oligopeptide. Injection of SLIGRL increased mean rolling leukocyte flux fraction from 34 ± 11 to 71 ± 24% ($p < 0.05$) and decreased mean rolling velocity from 63 ± 29 to 32 ± 2 μm/s ($p < 0.05$). No significant changes occurred with control peptide injection. To further evaluate the role of PAR2 in inflammatory responses, PAR2-deficient mice were generated by gene targeting and homologous recombination. Perivenular injections of SLIGRL resulted in only a small increase in rolling leukocyte flux fraction (from 21 ± 8 to 30 ± 2%) and no change in rolling velocity. Leukocyte rolling after surgical trauma was assessed in 9 PAR2-deficient and 12 wild-type mice. Early (0–15 min) after surgical trauma, the mean leukocyte rolling flux fraction was lower (10 ± 3 vs 30 ± 6%, $p < 0.05$) and mean rolling velocity was higher (67 ± 46 vs 52 ± 36 μm/s, $p < 0.01$) in PAR2-deficient compared with control mice. The defect in leukocyte rolling in PAR2-deficient mice did not persist past 30 min following surgical trauma. These results indicate that activation of PAR2 produces microvascular inflammation by rapid induction of P-selectin-mediated leukocyte rolling. In the absence of PAR2, the onset of inflammation is delayed. ***The Journal of Immunology,*** **2000, 165: 6504–6510.**

Accumulation of leukocytes in regions of inflammation depends on their recruitment and extravasation from the circulating blood pool. The initial step of recruitment is leukocyte capture and rolling along the endothelial surface of postcapillary venules, which permits firm leukocyte adhesion in the face of high shear rates and eventual transmigration through the vessel wall (1, 2). By virtue of slowing leukocyte transit through regions of inflammation, rolling may also enhance leukocyte exposure to proinflammatory cytokines and chemoattractants (3, 4). The process of leukocyte rolling is mediated primarily by the selectin family of adhesion molecules that share common structural features, including a similar NH_2-terminal lectin domain (5). E- and P-selectin are expressed on the endothelial cell surface in response to inflammatory stimuli and bind carbohydrate-bearing ligands on the leukocyte cell surface. L-selectin is constitutively expressed on most leukocyte types and binds ligands on the endothelium.

Induction of leukocyte rolling occurs rapidly in response to tissue injury or inflammation. Intravital microscopy has demonstrated leukocyte rolling in small venules several minutes following trauma induced by surgical exteriorization of the mouse cremaster muscle (6) or rat mesentery (7). This early rolling is almost entirely dependent upon recruitment of P-selectin to the luminal surface of vascular endothelial cells from preformed stores within the Weibel-Palade bodies (8). Mice deficient in P-selectin are characterized by deficient leukocyte rolling for up to 1 h after exteriorization of the cremaster muscle, after which E- and L-selectin-dependent rolling is observed (6, 8, 9).

Cell surface expression of P-selectin occurs in response to many stimuli, including cytokines, LPS, thrombin, and histamine (8, 10–12). Thrombin induces P-selectin expression by a cascade of events initiated by binding to the thrombin receptor, or protease-activated receptor-1 (PAR1),[4] on the endothelial cell surface (13). This receptor belongs to a family of unique G protein-coupled receptors that undergo proteolytic cleavage of the amino-terminal exodomain by serine proteases to produce a new amino terminus that functions as a tethered ligand to activate receptor function (14, 15). Proteolytic activation of PAR1 by thrombin results in activation of phospholipases A_2, C, and D (15, 16). Subsequent inositol triphosphate production and elevation of cytosolic Ca^{2+} (16) are most likely responsible for mobilization of granular stores of endothelial P-selectin (15). PAR1 agonists, such as the unmasked amino-terminal tethered ligand (SFLLRN), have been shown to promote P-selectin expression in cultured endothelial cells (17) and to induce P-selectin-dependent rolling in the mesentery of the rat (13).

Histamine is another vasoactive substance that triggers cell surface expression of P-selectin on cultured endothelial cells (12) and

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[4] Abbreviations used in this paper: PAR, protease-activated receptor; HMEC, human microvascular endothelial cell; LTC_4, leukotriene C_4; PAF, platelet-activating factor.

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induces leukocyte rolling in rat mesentery (7). Release of histamine upon degranulation of perivascular mast cells can induce P-selectin-mediated leukocyte rolling seen early after exteriorization of the mesenteric membrane in rats (7). Treatment with histamine receptor antagonists does not, however, abolish this rolling, suggesting that, in addition to histamine, other substances may mediate venular P-selectin expression in the setting of trauma or inflammation. One potential mast cell-derived product is tryptase, which activates another member of the protease-activated receptor family, PAR2. This receptor is structurally homologous to PAR1 with a proteolytic cleavage site located between arginine (Arg^{36}) and serine (Ser^{37}) residues near the amino terminus (18). Activation of PAR2 by tryptase, trypsin, or the hexapeptide agonist SLIGRL that corresponds to the murine tethered ligand sequence results in increases in intracellular Ca^{+}, similar to the response to PAR1 activation (16, 19, 20). Accordingly, it has been demonstrated that treatment of HUVECs with trypsin or the PAR2 peptide agonist SLIGRL results in cell surface expression of P-selectin and von Willebrand factor from Weibel-Palade bodies (21). The role of PAR2 in promoting inflammatory responses is further suggested by the demonstration that mRNA for PAR2 is up-regulated in HUVECs in response to IL-1, TNF-α, and LPS (22). More recently, superfusion of rat mesentery with PAR2 agonist peptides has been shown to increase leukocyte rolling and adhesion in venules (23).

In this study, we sought to further characterize the role of PAR2 receptor activation in initiating the inflammatory cascade. Early P-selectin-dependent leukocyte rolling was evaluated by intravital microscopy of the cremaster muscle of wild-type and PAR2-deficient mice generated by gene targeting and homologous recombination. Leukocyte rolling in venules was also assessed in wild-type and PAR2-deficient mice following perivascular microinjections of the PAR2 hexapeptide agonist SLIGRL, which possesses little cross-activity with PAR1 (19).

Materials and Methods

Generation of PAR2-deficient mice

A bacterial artificial chromosome containing the PAR2 gene (*Par2g*) was obtained by PCR screen of a 129/SvJ mouse genomic library (Genome Systems, St. Louis, MO). A 7-kb *Xho*I/*Hin*dII fragment of the Par2g intron and a 1.2-kb fragment of Par2g 3′ untranslated sequence generated by PCR (forward primer, 5′-TGGTAGGGATGCACCAACATG-3′ corresponding to nts 1379–1399 of GB Z48043; reverse primer, 5′-GGTAGGTTAAAG CAGACAGGGGAC-3′ corresponding to nts 2440–2463 of GB Z48043) were cloned into the pNTK vector to create the targeting vector (Fig. 1*A*). A 0.8-kb *Xba*I fragment of genomic sequence 3′ of the short arm of homology was used as a probe to identify both the wild-type and targeted alleles. RF8 embryonic stem cells (24) (129/SvJae) were electroporated with the targeting construct, and clones resistant to G418 and FIAU were selected and screened by Southern blot (Fig. 1*B*). A male mouse highly chimeric for $PAR2^{+/-}$ embryonic stem cells was bred to C57BL/6 females to generate $PAR2^{+/-}$ mice that were bred to generate $PAR2^{-/-}$ mice. Hemodynamic responses to the PAR2-activating peptide SLIGRL and control peptide LSIGRL were measured (25) and compared with control mice to confirm the $PAR2^{-/-}$ phenotype (Fig. 1*C*). Mice homozygous for a null mutation in the PAR2 gene were viable, fertile, of normal size and vitality, and did not show any signs of disease under specific pathogen-free vivarium conditions.

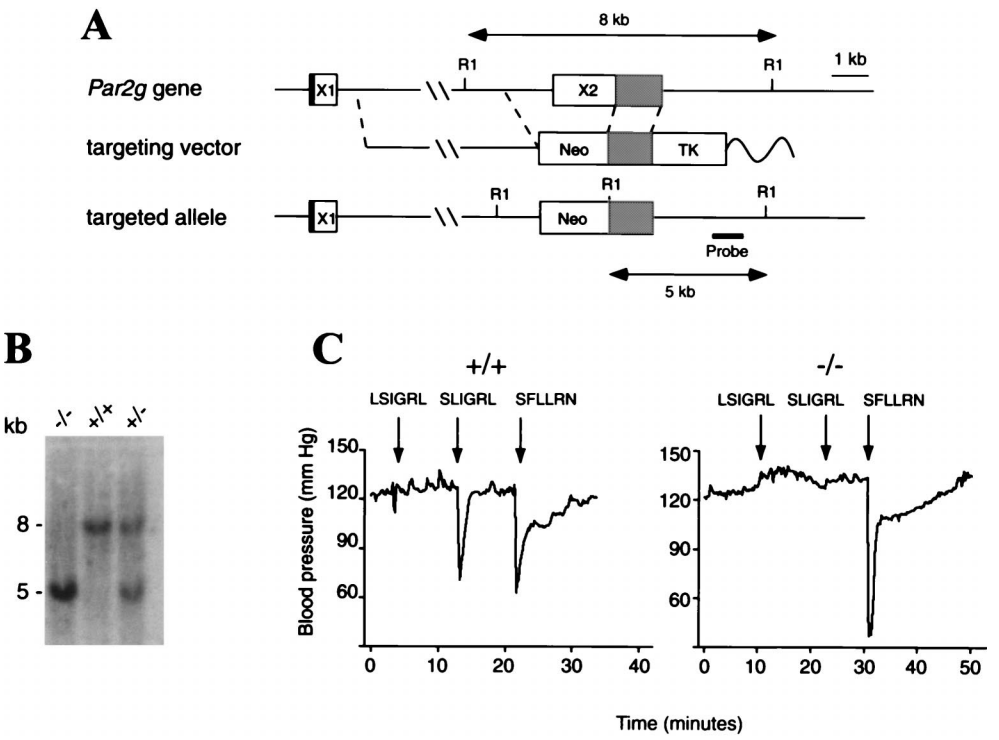

FIGURE 1. Generation of PAR2-deficient mice. *A*, Gene-targeting strategy. A replacement vector was used to substitute a neomycin phosphotransferase expression cassette (neo) for the entire PAR2 gene. Wavy line represents plasmid backbone; TK, HSV thymidine kinase expression cassette. X1 and X2 represent exons 1 and 2 of the PAR2 gene with the coding region shown as a white box and the 5′ and 3′ untranslated regions shown as shaded boxes. *B*, Southern blot analysis of *Eco*RI-digested genomic DNA from the tails of pups derived from $PAR2^{+/-}$ matings using 3′ flanking probe. Targeting introduced a new *Eco*RI site (R1). The 8- and 5-kb bands correspond to wild-type and targeted alleles, respectively. *C*, Loss of hemodynamic response to the PAR2-activating peptide SLIGRL in $PAR2^{-/-}$ mice. Wild-type (+/+) ($n = 6$) and $PAR2^{-/-}$ ($n = 7$) mice were catheterized, and aortic blood pressure was measured in the awake state. The control peptide LSIGRL, the PAR2-activating peptide SLIGRL, and the PAR1-activating peptide SFLLRN were injected to achieve an estimated concentration of 1 mM at the times indicated, and blood pressure was continually recorded.

Intravital microscopy

The study protocol was approved by the animal research committee at the University of Virginia. Twelve male PAR2-deficient ($PAR2^{-/-}$) mice and 14 littermate wild-type mice weighing between 24 and 32 g were anesthetized with an i.p. injection (12.5 μl/g) of a solution containing ketamine hydrocholoride (10 mg/ml), xylazine (1 mg/ml), and atropine (0.02 mg/ml). Body temperature was maintained at 37°C with a heating pad. A jugular vein was cannulated for administration of fluids and 0.1 mg pentobarbital approximately every 45 min as needed to maintain anesthesia. One carotid artery was cannulated for blood pressure monitoring. Blood samples were withdrawn from the carotid arterial catheter at the onset and conclusion of the experiment to analyze systemic leukocyte concentrations using hemocytometer (Fisher Scientific, Pittsburgh, PA) measurements of Kimura-stained samples.

Either the right or the left cremaster muscle was prepared for intravital microscopy, as previously described (6). The muscle was exteriorized through a scrotal incision and secured to a translucent pedestal. A longitudinal incision was made in the muscle, the edges were secured to the pedestal, and the epididymis and testicle were gently pinned to the side. The preparation was superfused continuously with isothermic bicarbonate-buffered saline.

Microscopic observations were made using an intravital microscope (Axioskop FS; Zeis, Oberkochen, Germany) with a saline immersion objective (SW 40/0.75 numerical aperture). Video recordings were made using a high-resolution camera (VE-1000CD, Dage-MTI) connected to an S-VHS recorder (Panasonic; Matsushita Electric Co.). Centerline venular RBC velocities were measured using a dual photodiode (26) and converted to mean blood flow velocities by multiplying by an empirical factor of 0.625 (27). Shear rates (γ_w) were determined as: $\gamma_w = 2.12(8V_b)/d$, where V_b is the mean blood velocity, d is the vessel diameter, and 2.12 is a correction factor for the shape of the velocity profile (28).

Venular diameters were measured off-line using video calipers. Freeze-frame advancing allowed tracking of individual rolling leukocytes over a distance of 30–100 μm. The total distance traveled was divided by the elapsed time to derive the mean rolling velocity. The number of rolling leukocytes (r_n) was determined by counting leukocytes crossing a line perpendicular to the vessel during 1 min. Leukocyte rolling flux fraction (F), which reflects the percentage of leukocytes passing through a venule that are rolling, was calculated by $F = r_n/(0.25\pi d^2 \cdot V_b \cdot 60 \cdot C_L)$, where d is vessel diameter, V_b is centerline blood velocity, and C_L is the systemic blood leukocyte concentration (6).

Eight wild-type and nine $PAR2^{-/-}$ mice were used to assess leukocyte rolling in response to surgical trauma (6). Intravital microscopic observations were initiated within 8 min of exterioration of the cremaster muscle in all animals. Venules with diameters between 25 and 40 μm were recorded under transillumination for ≥1 min each, followed by measurement of centerline blood velocity. Video recordings and velocity measurements were made in three to five different venules in each animal within 1 h after exterioration of the cremaster.

Peptide microinjection

Six wild-type and three $PAR2^{-/-}$ mice were studied to assess leukocyte rolling and adhesion in response to microinjections of a PAR2 agonist. Solutions (100 nM) containing either the murine PAR2-specific hexapeptide agonist SLIGRL (18, 19) or an inactive scrambled control peptide LSIGRL in normal saline were loaded into glass micropipettes, which were pulled from standard borosilicate glass with an outer diameter of 1 mm (Stoelting, Wood Dale, IL) on a vertical pipette puller (Stoelting). The blunt end of each pipette was fire polished by briefly passing the end through a flame. The tip of the pipette was then beveled using a micropipette grinder (model EG-40; Narishige, Greenville, NY) with a 0.3-μm abrasive foil (6775; AH Thomas). The pipettes were filled with ~10 μl of either SLIGRL or LSIGRL peptide solution. Tubing was threaded through the pipette holder and then secured on the end of the filled pipette. At its opposite end, the tubing was connected to a 10-ml syringe and three-way stopcock system. The pipette holder was inserted within a piezo-driven micromanipulator (model DC-3k Märzhäuser-Wetzlar). The pipettes were positioned adjacent (within 20 μm) to venules between 25 and 40 μm in diameter. Solutions were microinjected into perivenular tissue for 30 s. Video recordings and velocity measurements were made immediately before and 2 min following injection of either SLIGRL or LSIGRL, which were performed in random order. Up to five microinjections were performed per animal.

Peritoneal inflammation

Peritonitis was created in five wild-type and five $PAR2^{-/-}$ mice by i.p. injection of 1 ml of autoclaved 4% thioglycolate broth. At 4 h, mice were sacrificed and the peritoneum was lavaged with 5 ml of PBS containing 5 mM EDTA. Cell counts were made using hemocytometer measurements of Kimura-stained samples, and cellular differentials were assessed on Wright-stained smears.

Flow cytometry

Human microvascular endothelial cells (HMEC-1) (29) were grown to confluence in cell culture flasks. The growth medium consisted of MCDB 131 (Life Technologies, Rockville, MD), 10 ng/ml epidermal growth factor (Collaborative Biomedical Products, Bedford, MA), 1 μg hydrocortisone, 10% FBS, 1% penicillin, 1% streptomycin, and 10 mM L-glutamine. Human peripheral blood (2 ml) containing 10 U/ml heparin was added to flasks containing HMEC-1 cells or without cells (control). Either SLIGRL or an inactive control peptide (final concentration, 100 nM) was added to flasks with and without HMEC-1 cells, and the suspensions were incubated for 45 min at 37°C. Cells in the suspension were separately stained using PE-conjugated mAbs against L-selectin (DREG56; PharMingen, San Diego, CA) and Mac-1 (ICRF44; PharMingen). RBC were lysed with a solution of 0.15 M NH_4Cl, 0.01 M $NaHCO_3$, and 0.001 M disodium EDTA. Cells were washed with BSA/PBS and fixed in 1% paraformaldehyde/PBS. Samples (10^4 cells) were analyzed by flow cytometry (FACScalibur; Becton Dickinson, Mountain View, CA) gated to the characteristic forward and side scatter of granulocytes. Results are displayed as histograms of PE fluorescence.

Statistical methods

Data are expressed as means ± SD. Differences in hemodynamic parameters, leukocyte counts, and leukocyte rolling or adhesion parameters were compared by Student's t test. Interval comparisons of leukocyte rolling flux fraction at the different time points were made by repeated-measures ANOVA. Differences were considered significant at $p < 0.05$ (two sided).

Results

Hemodynamics parameters

A total of 64 venules was studied in wild-type mice and 54 venules in $PAR2^{-/-}$ mice for experiments investigating leukocyte rolling in response to surgical trauma. The mean venular diameters, venular blood velocities, and shear rates were similar between wild-type and $PAR2^{-/-}$ mice (Table I).

Time course of leukocyte rolling

Leukocyte rolling was present in the initial venule studied in all animals, even at the earliest time point studied (4 min after exteriorization of the cremaster muscle). Measurements of rolling leukocyte flux fraction were grouped into the time intervals of 0–15, 16–30, and >30 min after exteriorization of the cremaster muscle, providing an approximately even distribution of venules studied at each interval (Fig. 2). In the $PAR2^{-/-}$ mice, the mean leukocyte rolling flux fraction at 0–15 min was significantly lower compared with that in wild-type mice (12 ± 2 vs 30 ± 6%, $p < 0.05$). Although a significant reduction in leukocyte rolling flux fraction in the mutant mice persisted at 16–30 min (14 ± 3 vs 34 ± 8%, $p < 0.05$), no difference was found after 30 min. This was due to a relative increase in the flux fraction in the mutant mice (Fig. 2).

Table I. *Venular hemodynamic parameters in wild-type and $PAR2^{-/-}$ mice*

	Wild Type	$PAR2^{-/-}$
Venules (n)	64	54
Venular diameter (μm)	35 ± 7	33 ± 6
Blood velocity (mm · s^{-1})	1.9 ± 0.7	2.2 ± 0.8
Wall shear rate (s^{-1})	456 ± 199	552 ± 276

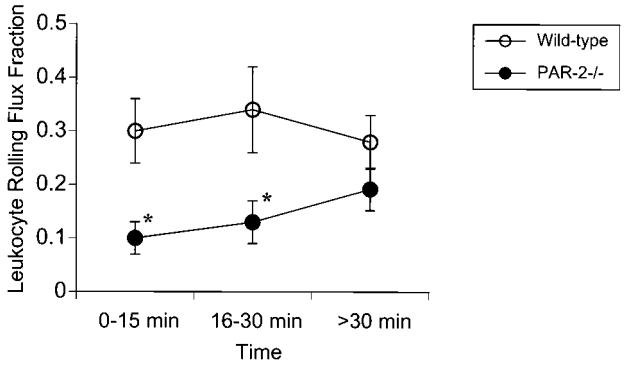

FIGURE 2. Mean (±SEM) leukocyte rolling flux fraction in cremasteric venules of wild-type and PAR2$^{-/-}$ mice. Rolling was assessed for up to 1 h. *, $p < 0.05$ compared with wild-type at the same time interval.

Rolling velocity was measured for a total of 646 leukocytes in wild-type mice and 427 leukocytes in PAR2$^{-/-}$ mice. The cumulative frequency histograms of leukocyte rolling velocities at the different time intervals (Fig. 3) illustrate that rolling velocity was significantly higher in PAR2$^{-/-}$ compared with wild-type mice only at the earliest time interval (0–15 min). At this time interval, the mean leukocyte rolling velocities in PAR2$^{-/-}$ and control mice were 67 ± 46 and 52 ± 36 μm/s ($p < 0.01$), respectively. In PAR2$^{-/-}$ mice, mean rolling velocity decreased with time such that there was no significant difference in rolling velocities after 15 min (Fig. 2).

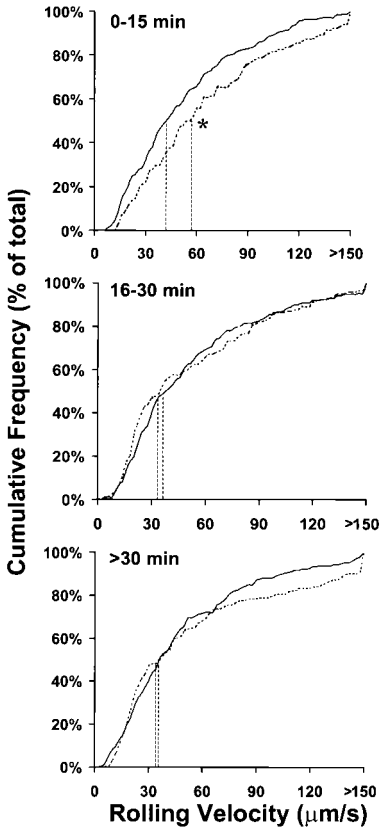

FIGURE 3. Cumulative frequency histograms of leukocyte rolling velocities in cremasteric venules of wild-type (solid lines) and PAR2$^{-/-}$ (dotted lines) mice. Vertical dashed lines illustrate median leukocyte velocities for each curve. *, $p < 0.05$ compared with the median for wild-type mice.

Leukocyte rolling and adhesion in response to agonist peptide

Perivenular microinjections of the active peptide agonist SLIGRL were performed in five and four venules in wild-type and PAR2$^{-/-}$ mice, respectively. Microinjections of the inactive control peptide LSIGRL were performed in four venules in wild-type mice. There were no differences in baseline vessel diameter, blood flow velocity, or shear rate between the different groups (Table II). Blood flow velocity and shear rate were unchanged 2 min following injection of either the active or control peptide (Table II). Examples of images obtained by intravital microscopy of a single venule in the cremaster muscle of a wild-type mouse immediately before and 2 min following perivascular injection of SLIGRL are shown in Fig. 4. These images demonstrate extensive leukocyte recruitment to the venular endothelial surface within 2 min of PAR2 activation. In wild-type mice, rolling leukocyte flux fraction more than doubled (from 34 ± 11 to 71 ± 24%, $p < 0.05$) in venules following treatment with SLIGRL, whereas flux fraction was unchanged with injections of the inactive peptide LSIGRL (Fig. 5*A*). A minimal increase in rolling leukocyte flux fraction (from 21 ± 8 to 30 ± 2%) was found in PAR2$^{-/-}$ mice following injection of SLIGRL (Fig. 5*A*).

Microinjections of SLIGRL in wild-type mice caused not only an immediate increase in leukocyte rolling, but also a significant reduction in mean leukocyte rolling velocity (from 63 ± 29 to 32 ± 2 μm/s, $p < 0.05$) (Fig. 5*B*), and an increase in the number of adhered leukocytes (Fig. 5*C*). The mean leukocyte rolling velocity and number of adhered leukocytes did not significantly change with microinjections of either the control peptide in wild-type mice or of SLIGRL in PAR2$^{-/-}$ mice (Fig. 5, *B* and *C*).

Peritoneal leukocyte recruitment

All mice survived to 4 h after induction of peritonitis. Total leukocyte counts in the peritoneal lavage at 4 h were not significantly different in wild-type compared with PAR2$^{-/-}$ mice (2.53 ± 0.49 vs 2.63 ± 0.85 [× 10^6] per ml, $p = 0.82$). The proportions of neutrophils, lympocytes, and monocytes/macrophages were also similar (data not shown).

Leukocyte activation by PAR2 agonist peptide

To investigate whether activation of PAR2 activates neutrophils, expression of L-selectin and Mac-1 was assessed using flow cytometry. Upon neutrophil activation, L-selectin is shed from the cell surface, resulting in reduced levels of expression (30), whereas Mac-1 surface expression is up-regulated (31). SLIGRL did not change expression of L-selectin or Mac-1 on neutrophils (Fig. 6, *top panels*). When SLIGRL was added to neutrophils incubated with HMEC-1 cells, L-selectin expression was reduced and Mac-1

Table II. *Venular hemodynamic parameters before and 2 min after microinjection of control or active PAR2 peptide agonist in wild-type and PAR2$^{-/-}$ mice*

	Wild Type		PAR2$^{-/-}$
	LSIGRL	SLIGRL	SLIGRL
Venules (*n*)	4	4	5
Venular diameter (μm)	30 ± 3	29 ± 3	33 ± 3
Blood velocity (mm · s^{-1})			
Preinjection	1.7 ± 0.3	1.3 ± 0.5	1.9 ± 0.5
Postinjection	1.7 ± 0.5	1.3 ± 0.5	2.0 ± 0.5
Wall shear rate (s^{-1})			
Preinjection	589 ± 148	441 ± 196	594 ± 289
Postinjection	595 ± 188	457 ± 220	605 ± 263

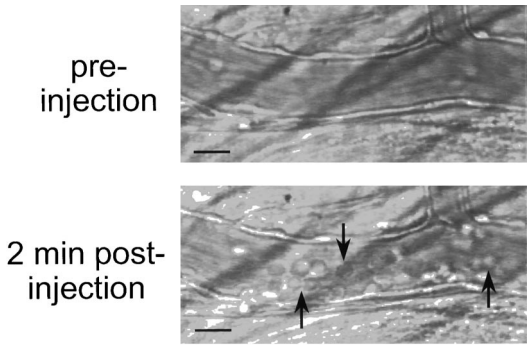

FIGURE 4. Microscopic images of a single venule from a wild-type mouse immediately before (*top panel*) and 2 min after (*bottom panel*) perivascular injection of SLIGRL. A marked increase in the number of rolling and adhered leukocytes (arrows) is seen following injection of the PAR2 agonist peptide. Scaling bar, 20 μm.

was up-regulated (*bottom panels*). This suggests indirect neutrophil activation via endothelial-derived mediators. Mac-1 and L-selectin expression were not affected by the inactive peptide LSIGRL (data not shown).

Discussion

PAR2-deficient mice showed decreased leukocyte rolling in a model of acute inflammation induced by mild tissue trauma. Conversely, in wild-type mice, activation of PAR2 by agonist peptide induced a significant reduction of leukocyte rolling velocity, increased leukocyte rolling flux, and increased leukocyte adhesion in wild-type mice. These findings suggest that PAR2 activation plays an important role in the induction of inflammatory responses.

A direct test of the in vivo effect of PAR2 activation was achieved by injecting a specific PAR2-activating peptide, SLIGRL, corresponding to the N terminus of the cleaved receptor (18). SLIGRL, but not control peptide, elicited three distinct responses in wild-type mice. First, the number of rolling leukocytes in small venules per unit of time, or rolling leukocyte flux, increased. Second, leukocyte rolling velocity decreased by about 50%. Third, a significant number of rolling leukocytes became firmly adherent. The first two observations are entirely consistent with increased surface expression of P-selectin in response to SLIGRL (21).

P-selectin is known to be up-regulated by surgical trauma caused by exteriorization of the cremaster muscle for intravital microscopy (7, 8). Leukocyte rolling in venules of the mouse cremaster muscle is largely P-selectin dependent for at least 60 min (6, 32). In this model, leukocyte rolling flux reaches a peak at about 50 min, which most likely corresponds to the peak of P-selectin expression (6). In the present study, peptide microinjections were performed after allowing the tissue to rest for at least 60 min after surgical exteriorization, a time when leukocyte rolling begins to decrease and P-selectin expression is less than maximal (6). The 2-fold increase in leukocyte rolling flux fraction with SLIGRL microinjection in this study is greater than that recently observed in rat mesenteric venules during superfusion with a PAR2 agonist (23). The greater response to the agonist in the current study most likely represents differences in both the dose and route of peptide administration as well as differences in the tissue and species studied.

A reasonable explanation for increased leukocyte rolling flux and decreased rolling velocity in response to SLIGRL is that activation of PAR2 increases surface expression of P-selectin on endothelial cells (13, 21). Endothelial cells are known to express

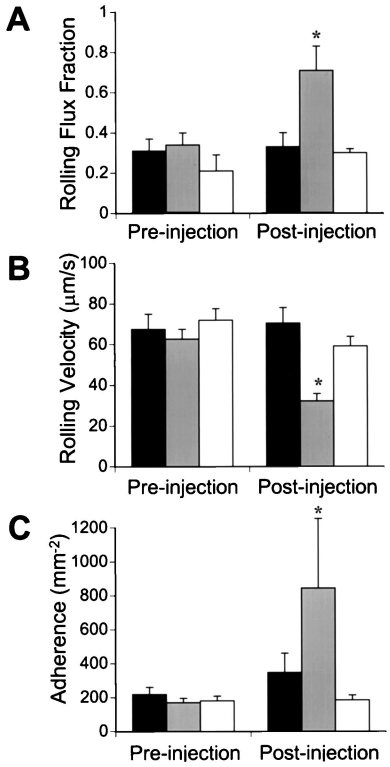

FIGURE 5. Mean (±SEM) leukocyte flux fraction (*A*), leukocyte rolling velocity (*B*), and number of adhered leukocytes (*C*) in cremasteric venules before and after perivascular microinjections in of inactive control peptide (LSIGRL) in wild-type mice (filled columns), active peptide agonist (SLIGRL) in wild-type mice (shaded columns), or active peptide in $PAR2^{-/-}$ mice (open columns). *, $p < 0.05$ compared with preinjection value.

PAR2 and respond to receptor activation by a rise in intracellular free calcium and activation of phospholipase C (16). Moreover, PAR2 activation by the peptide, SLIGKV, causes mobilization of intracellular free calcium, degranulation of Weibel-Palade bodies, and surface expression of von Willebrand factor in HUVEC (33). Since both von Willebrand factor and P-selectin are stored in Weibel-Palade bodies (34), it is likely that P-selectin expression is induced through the same mechanism. The conclusion that PAR2 activation induced increased surface expression of endothelial P-selectin is further supported by the similarity of the in vivo responses to PAR2 activation and to histamine (35) or leukotriene C_4 (LTC_4) (36), interventions that are known to cause acute up-regulation of P-selectin surface expression in endothelial cells (33, 37). The observed reduction in leukocyte rolling velocity following injection of SLIGRL is also consistent with increased expression of P-selectin. Our data obtained after activation of PAR2 parallel those seen previously in response to LTC_4, which caused a 50% reduction in leukocyte rolling velocity due to increased P-selectin expression (36).

The increase in leukocyte adhesion with SLIGRL is not readily explained by increased P-selectin expression. Even at high site densities, P-selectin does not support firm adhesion (38). However, endothelial stimulation with SLIGRL may induce endothelial cells to express other adhesion molecules and mediators relevant to leukocyte trafficking. For example, endothelial cell activation with histamine, thrombin, or LTC_4 all cause expression of platelet-activating factor (PAF) (37, 39). PAF is known to activate rolling leukocytes in a juxtacrine fashion (39, 40), resulting in activation of β_2 integrins that promote firm leukocyte adhesion. Leukocyte

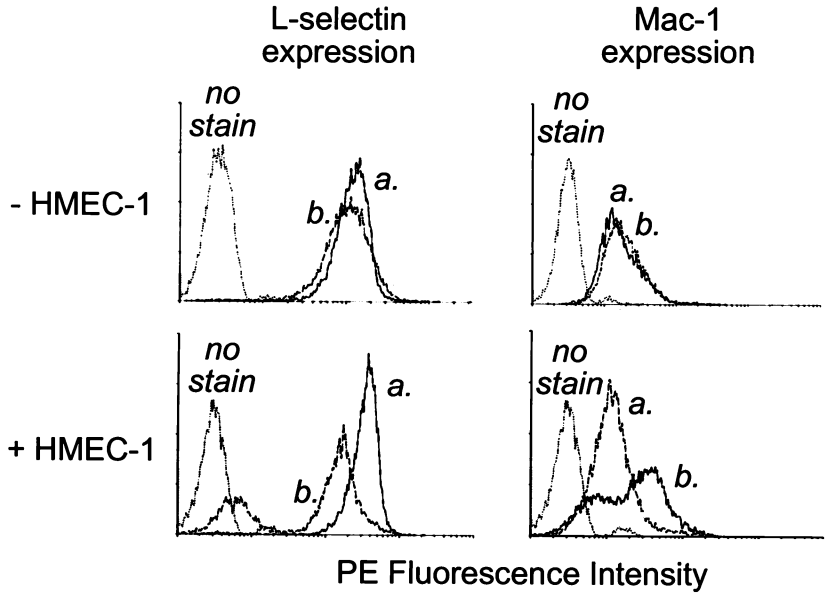

FIGURE 6. Flow cytometry data of L-selectin and Mac-1 expression on human granulocytes. Compared with experiments in which no peptide was added (a); addition of SLIGRL (b) resulted in L-selectin shedding and increased Mac-1 expression only when endothelial cells (HMEC-1) were present.

migration into the peritoneal cavity of rats following i.p. injection of PAR2 agonists has been shown to be inhibited by pretreatment with the PAF receptor antagonist WEB 2086 (23). Although endothelial cells can produce PAF (37, 39), and PAF receptor antagonist prevents neutrophil recruitment in response to PAR2 agonist (23), it is not clear whether endothelial cells produce a relevant neutrophil-activating signal in response to PAR2 activation. Accordingly, we investigated whether rapid leukocyte adhesion observed following SLIGRL microinjection is mediated by endothelial-derived mediators. Our flow cytometry data demonstrate leukocyte activation when exposed to SLIGRL and cultured endothelial cells, but not to SLIGRL or endothelial cells alone. This finding is consistent with PAR2-induced expression of PAF and possibly other mediators that can activate rolling leukocytes to promote firm adhesion. Although PAR2 has been reported to be present on neutrophils (41), we did not find evidence of direct neutrophil activation by SLIGRL as assessed by β_2 integrin expression or L-selectin shedding measured by flow cytometry.

The generation and characterization of a PAR2 null mouse enabled us to study the impact of this receptor on the inflammatory response. Previous descriptions of the effects of a different null mutation in the PAR2 gene did not address the impact of the absence of PAR2 on inflammatory responses (42, 43). In this study, we show that leukocyte rolling is markedly attenuated in PAR2$^{-/-}$ mice, indicating that PAR2 makes a substantial contribution to the initial inflammatory response seen in wild-type mice. The rolling defect was not sustained beyond 30 min after exteriorization, a time point at which P-selectin-independent mechanisms of rolling become important (6). The absence of a sustained defect in leukocyte recruitment explains similar peritoneal inflammatory responses in wild-type and PAR2$^{-/-}$ mice 4 h following thioglycolate injection.

P-selectin-dependent rolling in venules of the mouse cremaster muscle and similar intravital microscopic preparations is induced by mast cell degranulation, and prevented by inhibition of mast cell degranulation (8, 44). Mast cells contain and, upon stimulation, release tryptase, a known activator of PAR2 (20). Mast cells also contain and release histamine, which also can induce P-selectin expression on endothelial cells (32). However, blocking histamine H1 or H2 receptors or both does not reduce trauma-induced leukocyte rolling (7, 8, 44), although histamine application can induce leukocyte rolling (7). This suggests that histamine is not the only and maybe not even the most important mediator released by mast cells that can activate endothelial cells. The present data suggest that mast cell-derived tryptase may be an important and early activator of endothelial cells in response to tissue trauma. In addition to sensing tryptase, PAR2 can be activated by the tissue factor/factor VIIa complex and by factor Xa (45). The ability of PAR2 to sense activation of the coagulation cascade provides another potential link between tissue trauma and cellular responses. PAR2 activation has been reported to promote vasodilation and sensitivity to endotoxin (46) as well as neurogenic inflammation (47). Our data show that PAR2 is an important receptor in inflammation and, indeed, appears necessary for some of the earliest inflammatory responses in vivo.

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