Control of neutrophil pseudopods by fluid shear: role of Rho family GTPases

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Makino, Ayako, Michael Glogauer, Gary M. Bokoch, Shu Chien, and Geert W. Schmid-Schönbein. Control of neutrophil pseudopods by fluid shear: role of Rho family GTPases. Am J Physiol Cell Physiol 288: C863–C871, 2005. First published November 24, 2004; doi:10.1152/ajpcell.00358.2004.—Blood vessels and blood cells are under continuous fluid shear. Studies on vascular endothelium and smooth muscle cells have shown the importance of this mechanical stress in cell signal transduction, gene expression, vascular remodeling, and cell survival. However, in circulating leukocytes, shear-induced signal transduction has not been investigated. Here we examine in vivo and in vitro the control of pseudopods in leukocytes under the influence of fluid shear stress and the role of the Rho family small GTPases. We used a combination of HL-60 cells differentiated into neutrophils (1.4% dimethyl sulfoxide for 5 days) and fresh leukocytes from Rac knockout mice. The cells responded to shear stress (5 dyn/cm2) with retraction of pseudopods and reduction of their projected cell area. The Rac1 and Rac2 activities were decreased by fluid shear in a time- and magnitude-dependent manner, whereas the Cdc42 activity remained unchanged (up to 5 dyn/cm2). The Rho activity was transiently increased and recovered to static levels after 10 min of shear exposure (5 dyn/cm2). Inhibition of either Rac1 or Rac2 slightly but significantly diminished the fluid shear response. Transfection with Rac1-positive mutant enhanced the pseudopod formation during shear. Leukocytes from Rac1-null and Rac2-null mice had an ability to form pseudopods in response to platelet-activating factor but did not respond to fluid shear in vitro. Leukocytes in wild-type mice retracted pseudopods after physiological shear exposure, whereas cells in Rac1-null mice showed no retraction during equal shear. On leukocytes from Rac2-null mice, however, fluid shear exerted a biphasic effect. Leukocytes with extended pseudopods slightly decreased in length, whereas initially round cells increased in length after shear application. The disruption of Rac activity made leukocytes nonresponsive to fluid shear, induced cell adhesion and microvascular stasis, and decreased microvascular density. These results suggest that deactivation of Rac activity by fluid shear plays an important role in stable circulation of leukocytes.

Shear stress due to blood flow greatly influences vascular structure and the inflammatory response. These phenotypic alterations resulting from shear stress have been examined mostly in vascular endothelial and smooth muscle cells and involve a coordinated sequence of events with rapid activation of flow-sensitive K+ and Cl- channels (34, 40) and GTP-binding proteins (G proteins) (18, 29, 53), changes in cell membrane fluidity (5, 19) and intracellular pH (58), and mobilization of intracellular calcium (2, 25, 47). The early responses are followed by activation of a host of gene and protein regulatory responses (32, 39, 42) and by cytoskeletal remodeling (11, 35, 53).

We have obtained increasing evidence suggesting that fluid shear stress also affects circulating cells, such as leukocytes. The leukocyte-endothelial cell adhesion is a critical step during immune responses against bacterial infection and the inflammatory cascade. The binding of circulating leukocytes to the endothelium is regulated by fluid shear (8, 24, 30). Fluid shear controls the morphology of neutrophils, but different from that of endothelial cells or smooth muscle cells, and it controls the cytoplasmic properties as well as the distribution of membrane adhesion molecules (12, 37, 48).

The molecular details of cytoskeletal reorganization induced by fluid shear stress in leukocytes, however, are unexplored. This is the focus of the current report. The Rho family small GTPases, which are members of the Ras superfamily of small GTP-binding proteins, have been shown to play an essential role in a variety of cellular functions, including the regulation of actin cytoskeleton, membrane trafficking, gene transcription, oxidant generation, cell growth, chemotaxis, and cell adhesion (20). Neutrophils have the ability to project thin membrane-covered cytoplasm regions called pseudopods. There are several general shapes, including veil-like lamellipodia, finger-like projections, filopodia, and uropodia during pseudopod retraction. Pseudopod formation is mediated by several signaling pathways, including the Rho family GTPases. Activated Cdc42 and Rac stimulate the Arp2/3 complex via Wiskott-Aldrich syndrome protein (WASP) and WASP-family verprolin protein, respectively (23, 51). The activated Arp2/3 complex serves as an actin-nucleating factor. Cdc42 forms actin-rich filopodia and Rac stimulates the formation of lamellipodia and membrane ruffles (20). Although stress fibers or focal adhesion sites are not formed in leukocytes, it has been reported that RhoA regulates uropod detachment by stimulating actomyosin filaments (1, 54).

Thus we examine here the small GTPase activity in neutrophils during shear-induced pseudopod retraction. We use fresh leukocytes and human promyelocytic HL-60 cells differentiated by dimethyl sulfoxide (DMSO) into neutrophil-like cells (9, 22). Because appropriate expression vectors and transfection methods have been developed that allow expression of exogenous proteins in HL-60 cell line (41), we use these cells as part of the in vitro experiments.

We show that Rac1 and Rac2 make an important contribution to the fluid shear response as well as to the adhesion to endothelial in postcapillary venules.

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MATERIALS AND METHODS

Biological materials and reagents. Human promyelocytic leukemic HL-60 cells from the American Type Culture Collection were cultured in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Omega Scientific) in 5% CO2 at 37°C. HL-60 cell differentiation was induced by treatment with 1.4% DMSO (American Type Culture Collection) for 5 days. The rabbit anti-Rac2 polyclonal antibody, GST-PBD, GFP-tagged T17NRac1, T17NRac2, Q16LRac1, and Q16LRac2 have been described previously (4). The mouse anti–Rac1 monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), and the Rho Activation Assay Kit and mouse anti-cdc42 monoclonal antibody were obtained from Upstate Biotechnology (Waltham, MA). The protease inhibitors, PMSF, aprotinin, and leupeptin, and the chemoattractant, formyl-methionyl-leucyl-phenylalanine (fMLP), and platelet-activating factor (PAF) were from Sigma (St. Louis, MO).

Transfection of HL-60 cells. HL-60 cells were cultured in the presence of 1.4% DMSO for 4 days to initiate differentiation before transfection. On day 4, the cells were washed and suspended in Opti-MEM reduced serum medium (Invitrogen) at a cell concentration of 2 × 10^6 cells/well. cDNA (4 μg) were suspended in Opti-MEM and mixed with the cationic lipid 1,2-dimyristyloxypropyl-3-dimethyldihydroxethyl ammonium bromide/cholesterol (Invitrogen, 4 μl/well). The cationic lipid/cDNA mixture was added to the cells and incubated at 37°C for 4 h. An equal volume of RPMI 1640 medium containing 20% heat-inactivated fetal bovine serum plus DMSO (1.4% final concentration) was then added, and the cells were cultured for 20 h. On day 5, the cells were washed and suspended in fresh RPMI that contained 10% FBS and were cultured for an additional 24 h before the experiment.

Animals. Conditional Rac1 knockout (Rac1^1/− LysM^−/−) mice had previously been generated by targeted disruption of the rac1 gene in neutrophils (16). All experiments were compared in neutrophils isolated from Rac1^1/−LysM^−/− mice and Rac1^+/−LysM^−/− mice (45) were backcrosses (>12 generations) into C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME). The mice used in these experiments were 9–17 wk of age. All animal procedures were approved by the University of California, San Diego, Animal Subject Committee.

Marine leukocyte isolation. Whole blood leukocytes and enriched neutrophil populations were isolated from whole blood of anesthetized mice (50 mg/kg ip pentobarbital sodium). Anticoagulated blood samples (10 U/ml ammonium heparin) were collected by cardiac puncture using a 1-ml syringe with a 23-G needle. For whole blood leukocytes, a leukocyte-rich buffy coat was obtained by centrifugation at 200 g for 5 min at 4°C. Sporadic red blood cells were removed via hypotonic lysis performed at 4°C. The mixture of platelets and leukocytes was immediately resuspended with RPMI 1640 containing 10% FBS.

Human leukocyte isolation. Fresh leukocytes from finger prick blood of volunteers were collected in capillary hemocrit tubes (ammonium heparin prefilled; Clay Adams). The red blood cells were allowed to sediment at room temperature for 1 h. The supernatant mixture of platelets and leukocytes was resuspended in RPMI 1640 containing 10% FBS.

Fluid shear application in a flow chamber. The differentiated HL-60 cells or mouse leukocytes were subjected to laminar shear flow in a rectangular flow channel sandwiched with a gasket (230 μm thick) between a coverslip and a glass plate (15). The chamber was mounted on the stage of an inverted phase-contrast microscope (model IX70, Olympus, Japan). All flow experiments were carried out at 24°C. Cells were suspended in RPMI 1640 containing 10% FBS and loaded into the flow chamber at a slow flow rate with wall shear stress lower than ~1 dyn/cm². Laminar shear stress was generated by perfusion of HBSS (pH 7.4 adjusted with 100 μmol/l HEPES, containing Ca²⁺ and Mg²⁺ at 1 mmol/l each, Life Technologies) with an automated syringe pump (Harvard Apparatus) attached to the inlet side of the flow chamber.

Images of the cells were recorded via a ×60 objective and a ×1.6 eyepiece with a charge-coupled device camera (model VI-470, Optronics) and stored digitally (Scion Image Software). Images were recorded during 10 min of fluid shearing and for 10 min during the recovery phase. Each cell contour was outlined manually and the area was computed (Scion Image Software) (Fig. 1A). This measurement will be referred to as projected cell area. Some of the results are shown as normalized cell area (projected cell area divided by the average of the projected cell area during the control period before shear).

Fluid shear application in a cone-and-plate device. We used the cone-and-plate device to collect sufficient concentrations of protein for the pull-down assay to measure small GTPase activity. This approach permits the use of suspended cells. But the flow and shear...
stress pattern on the leukocyte membrane is different from that in the parallel plate flow chamber (49). One main difference between the two situations is that leukocytes in free suspension experience on their membrane higher rates of change of shear stress with respect to time compared with cells adhering to a substrate surface, e.g., in the flow chamber. Leukocytes in the circulation obviously experience both situations as they are carried in the free circulation to a position where they adhere to the endothelium.

We suspended 10^7 cells with 0.5 ml of HBSS and subjected to shear stress in the cone-and-plate device (model DV II, Brookfield, Middleboro, MA). The device consists of a rotating 0.8° cone placed over a stationary plate. The gap between the cone and plate at the center is 12.5 μm. The experimental apparatus was maintained at 24–25°C. To apply fluid shear stress, the cell suspensions were placed in the gap between the cone and plate. The cone turned at a constant rotational speed, generating a uniform fluid shear field to the entire cell suspension.

Rac, cdc42, and Rho pull-down assays. Rac and cdc42 activation assays were performed as described (4). Immediately after the cessation of shear in the cone-and-plate device, an equal volume of lysate buffer (0.5 ml; 100 mmol/l Tris-HCl, pH 7.4, 4 mol/l NaCl, 2 mmol/l MgCl2, 1% Igepal, 20% glycerol, 2 mol/l EGTA, 20 μg/ml leupeptin, 20 μg/ml apronitin and 2 mol/l PMSF) was added to arrest cell activation. The cell lysate was centrifuged and 600 μl of the supernatant was incubated with PAK1-PBD agarose (10 μg) for 1 h at 4°C, washed three times, and eluted with SDS sample buffer. Total Rac or cdc42 proteins were measured using 10 μl of lysate. Rac1, 2, and cdc42 proteins were detected with Western blot analysis using anti-Rac1 mouse monoclonal antibody (dilution 1:2,000), anti-Rac2 rabbit polyclonal antibody (1:2,000), and anti-cdc42 mouse monoclonal antibody (1:1,000), respectively. To measure the activation of Rho, the cells were lysed in 500 μl of lysate buffer and 800 μl of the lysate were incubated with Rhotekin RBD agarose (20 μg, Upstate Biotechnology) following the manufacturer’s recommendations. The total Rho present in 10 μl of the lysate was measured before incubation with Rhotekin RBD. Total Rho and activated Rho bound to Rhotekin-RBD were detected by Western blot analysis with the use of anti-RhoA mouse polyclonal antibody (1:2,000). The immunoblots were detected using the ECL Western blotting detection reagents (Amer-sham Biosciences). Densitometric analysis was performed using Scion Image software. The densitometric quantification was attained from the ratio of the active form and the total GTPase protein.

Surgical procedure for intravital microscopy. Male mice were anesthetized with pentobarbital sodium (50 mg/kg ip). Anesthesia was maintained with supplemental doses of pentobarbital sodium (5 mg/kg iv) as needed. The left femoral vein was cannulated for the administration of supplemental anesthesia. Mice were placed in a dorsal recumbent position on a microscope stage, maintained at 37°C with an optically clear viewing pedestal, and surgically prepared. Briefly, an incision was made in the scrotal skin to expose the left cremaster muscle, which was then carefully removed from the associated fascia. A lengthwise incision was made on the ventral surface of the cremaster muscle using cautery. The testicle and the epididymis were separated from the underlying muscle and moved into the abdominal cavity. The muscle was then spread over an optically clear view cavity. The muscle was then spread over an optically clear view chamber. Before shear, the cells were migrating on the cover-slip and spreading their cytoplasm. Fluid shear stress induced a rapid and significant reduction of projected cell area in the differentiated HL-60 cells. During shear, the cells had a smaller projected area with reduced cytoplasmic spreading. After cessation of fluid shear, there was no significant recovery of cell spreading over a 10-min period (Fig. 1B). In line with HL-60 cells, application of fluid shear to adherent human neutrophils caused significant retraction of projected pseudopods and also the recovery of cell area was not seen during 10 min after shear application (Fig. 1C).

Shear stress controls small GTPase activity in a time- and magnitude-dependent manner. To assess whether physiological shear stress in a buffer solution (5.2 dyn/cm^2 at a shear rate of 450 s^{-1}) affects the activity of the Rho family small GTPases, we applied fluid shear to differentiated HL-60 cells in the cone-and-plate device. The sheared cells were immediately lysed and subjected to a pull-down assay to detect the active forms of small GTPases. Rac1 and Rac2 activities were significantly decreased by fluid shear stress applied for 5 min, whereas cdc42 exhibited no significant change at any time point up to 10 min (Fig. 2A). RhoA activity was transiently

**RESULTS**

Differentiated HL-60 cell and human neutrophil response to fluid shear. To examine how differentiated HL-60 cells respond to fluid shear, we applied a shear stress (5 dyn/cm^2 for 10 min) to the cells while they adhered to the wall of the flow chamber. Before shear, the cells were migrating on the coverslip and spreading their cytoplasm. Fluid shear stress induced a rapid and significant reduction of projected cell area in the differentiated HL-60 cells. During shear, the cells had a smaller projected area with reduced cytoplasmic spreading. After cessation of fluid shear, there was no significant recovery of cell spreading over a 10-min period (Fig. 1B). In line with HL-60 cells, application of fluid shear to adherent human neutrophils caused significant retraction of projected pseudopods and also the recovery of cell area was not seen during 10 min after shear application (Fig. 1C).

**Micropipette perfusion.** The foraminiferan was observed in the unbranched cremaster venules (25–40 μm in diameter). Platelet-activating factor (PAF; ~10^{-9}–10^{-8} M) was superfused to allow cells to project pseudopods. Flow stress with near zero shear stress was induced for 3–5 min by obstruction at upstream locations of venular segments with a micropipette. The micropipette was then removed and the venule was returned to physiological shear stress for 5 min. Leukocytes that remained on the venular endothelium after shear restoration but did not adhere to the wall for >30-s period were used to determine the cell length. L refers to maximum length across the cell (cell body plus pseudopod), and L0 refers to the diameter of the cells in its spherical state without pseudopods.

Another set of mice was used for determining the number of adherent cells. A leukocyte was considered adherent to the venular endothelium if it remained stationary within a preselected area of venule for 30 s. After the experiments, cremaster muscle samples were stained with FITC-labeled Bandeiraea simplicifolia lectin (Sigma) to outline the microvascular bed. Fluorescence images were obtained using confocal microscopy (excitation/emission = 488/515; model MRC-1024UV, Bio-Rad), and they were digitized and quantified using an automated computer vessel-counting method previously described (43).

To measure the wall shear stress in the postcapillary venule, 0.5 ml of fluorescently labeled erythrocytes from donor mouse (DiIC16, Molecular Probes) was intravenously injected. The velocities of 75 labeled erythrocytes in each venule were measured using an intravital fluorescence microscope with a silicone-intensified target camera (DAGE-MTI). The wall shear stress on the endothelium in each venule was estimated as 4 × mean velocity × plasma viscosity/ internal radius of the vessel, according to flow at a low Reynolds number in a cylindrical blood vessel.

**Statistical analysis.** Measurements are presented as means ± SE. For statistical comparison, two-way ANOVA with repeated measurements was carried out, and the Bonferroni (Dunn) test was used as a post hoc test. Comparisons between groups of the Western blot data were carried out by unpaired Student’s t-test.

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increased after 2 min of shear and returned to its control level at 10 min (Fig. 2A). On the basis of these results, we examined the effect of the shear stress magnitude on the small GTPase activities. We applied fluid shear at different magnitudes (0, 1.2, 2.5, and 5.2 dyn/cm²) to the differentiated HL-60 cells for 10 min (in the cases of Rac and cdc42 activities) or for 2 min (in the case of Rho activity) (Fig. 2B). A shear stress at 5.2 dyn/cm² caused a significant reduction in Rac1 and Rac2 activities, no significant change in cdc42 activity, and a marked enhancement of the RhoA activity. 

Shear stress-induced reduction in projected cell area is associated with deactivation of Rac1 and Rac2. To investigate the role of Rac in fluid shear-induced reduction of the projected area of differentiated HL-60 cells, we tested whether positive or negative Rac mutant affects the cellular response to shear stress (Fig. 3). GFP fused to dominant negative (T17N) or positive (Q61L) Rac was transiently transfected into the differentiated HL-60 cells. cDNA encoding for only GFP was transfected into the cells as a control. T17N Rac1 weakened the shear stress-induced reduction in projected cell area, whereas Q61L Rac1 caused an increase in the projected cell area after shear application. Rac2 mutants blocked the shear-induced reduction in cell area.

fMLP increased small GTPase activities, inhibited shear stress-induced deactivation of Rac1 and Rac2 and blocked the cell response to fluid shear. fMLP, which increases small GTPase activities and enhances the migration of human neutrophils (4, 7, 57), was used to test whether it may affect the shear stress-induced small GTPase activities (Fig. 4). The differentiated HL-60 cells were suspended in HBSS with fMLP (10⁻⁹ M) and sheared in the cone-and-plate device. Each GTPase activity was measured in unsheared (static) and sheared (5.2 dyn/cm² for 10 min) cells in the absence or presence of fMLP. Under static conditions, fMLP markedly increased the activities of all three GTPases. The shear stress-induced downregulation of Rac activity (Fig. 2) was abolished by fMLP pretreatment, and actually caused a significant increase in Rac2 activity above that under static condition (Fig. 4A). fMLP pretreatment also caused significant increases in cell spreading as measured by the projected cell area of differentiated HL-60 cells. Real-time images of the cells suspended in culture medium with fMLP (10⁻⁹ M) also confirm...
that the projected cell area does not decrease during fluid shear (Fig. 4B).

Leukocytes from both Rac1-null mice and Rac2-null mice do not exhibit shear-induced pseudopod retraction in vitro. Leukocytes from Rac-deficient mice exhibited enhanced spreading activity in the resting state (spreading cells: wild type, 2.38 ± 1.8%; Rac1-null, 7.46 ± 1.8%; Rac2-null, 7.18 ± 3.9%), and over half of the cells from each group maintained the ability to form pseudopods in response to 5 × 10⁻⁸ M PAF (cells with small pseudopods/spreading cells: wild type, 59.9 ± 3.4%; Rac1-null, 51.6 ± 5.3%; Rac2-null, 59.8 ± 5.1%) (Fig. 5A).

The response of leukocytes, which had pseudopods without stimulation, to fluid shear was examined in the flow chamber. Fluid shear (5 dyn/cm²) induced a reduction of cell area in wild-type leukocytes, whereas cells taken from either Rac1-null or Rac2-null mice not only lost their response to the fluid shear but also markedly increased their cell area during shear exposure (Fig. 5B).

In vivo leukocyte response of Rac-deficient mice to physiological shear. Selected postcapillary venules were occluded by a micropipette for 3–5 min, during which time the leukocytes were allowed to form pseudopods. After occlusion release, the cells were exposed to physiological shear stress (12.6 ± 0.7 dyn/cm²). We encountered a wide variety of cell length responses and classified them into two groups according to their initial values, i.e., initially more extended (L/L₀ > 1.4) and less extended (L/L₀ ≤ 1.4) cells. This approach is optimal to summarize the different cell responses we encountered.

Both more and less extended cells in wild-type mice reduced their length during fluid shear. In contrast, cells from Rac1-null mice exhibited no change of their cell shape during shear. In Rac2-null mice, cells that were initially more extended decreased their length but did not reach a round shape. In contrast, cells that were initially less extended increased their length in response to fluid shear (Fig. 6).

Rac-deficient mice exhibited vascular stasis in response to PAF and had lower microvascular density than wild-type mice. Compared with the wild-type mice, Rac1-null and Rac2-null mice had significantly lower body weight. After treatment with PAF, both forms of Rac-deficient mice significantly increased the number of leukocytes adhering to, and readily induced stasis in, the postcapillary venules (Table 1). Rac1-null mice...
had a lower microvascular density in their cremaster vascular bed compared with wild-type mice.

DISCUSSION

Previous in vitro studies suggest that human neutrophils in an activated state respond to fluid shear by pseudopod retraction (14, 37, 48), whereas spherical leukocytes without pseudopods may actually project pseudopods when exposed to fluid shear (10). Trigger signals to induce pseudopod retraction or projection may involve calcium influx and nitric oxide production (14, 37). We demonstrate here that the shear-induced signaling pathway is mediated by Rho family small GTPases.

Specifically, Rac controls shape adjustment of leukocytes in response to fluid shear in vitro and in vivo. Physiological fluid shear induces retraction of lamellipodia with a reduction of the projected cell area in human neutrophils as well as in differentiated HL-60 (Fig. 1). The cell responses we see here are all evoked by physiological but small fluid shear stresses (5 dyn/cm² is ~5/981 cmH₂O) far below the values needed to achieve a passive viscoelastic response (~100 to 1,000 dyn/cm²), representing a short-term reaction compared with the majority of longer-term shear responses reported for endothelial cells or smooth muscle cells. The current studies show the response for a period of <10 min, a short period compared with the typical 1 to 24 h required to establish steady state elongation of endothelial cells (in the direction of fluid shear) or smooth muscle cells (perpendicular to fluid shear) (6, 31, 52, 53).

A key concern was the use of different measures to quantify the rather complex shape changes encountered in leukocytes forming pseudopods. In pilot studies, we experimented with three different morphological parameters to describe the cell response to shear stress; viz., projected cell area, longest length across the cell, and a cell shape index S ([cell area]²/[perimeter length]/4π) (data not shown). Because the cells have small pseudopods, the longest length across the cell or the shape index were not particularly sensitive indicators. Instead, the projected cell area was found to be the most sensitive index for detecting the morphological response to shear in vitro, and it was used in this analysis.

Neutrophil motility depends on the cycling of actin protein subunits between monomeric and polymeric pools and the reversible cross-linking of the polymeric actin into three-dimensional networks. There are multiple mechanisms for the control of the actin state. These mechanisms are not mutually

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Table 1. Phenotype of knockout mice

<table>
<thead>
<tr>
<th>Phenotype of knockout mice</th>
<th>Wild Type</th>
<th>Rac1-Null</th>
<th>Rac2-Null</th>
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</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>28.2±0.7 (12)</td>
<td>25.3±0.6*(11)</td>
<td>26.1±0.5*(10)</td>
</tr>
<tr>
<td>Microvascular density, mm/mm³</td>
<td>1951±232 (5)</td>
<td>1411±97*(6)</td>
<td>1641±146 (5)</td>
</tr>
<tr>
<td>Adherent cells, cells/80 µm</td>
<td>0±0 [12]</td>
<td>2±1 [18]</td>
<td>1±0 [13]</td>
</tr>
<tr>
<td>PAF (10⁻⁷ M)</td>
<td>1±0 [4]</td>
<td>26±5* [10]</td>
<td>16±2* [9]</td>
</tr>
<tr>
<td>Time to stasis in postcapillary venules, min</td>
<td>&gt;120 (5)</td>
<td>&gt;120 (4)</td>
<td>&gt;120 (4)</td>
</tr>
<tr>
<td>PAF (10⁻⁷ M)</td>
<td>&gt;120 (4)</td>
<td>93±16* (4)</td>
<td>96±9* (5)</td>
</tr>
</tbody>
</table>
| Values are means ± SE. PAF, platelet-activating factor. Parentheses show the number of mice. Brackets show the number of observed areas. Adherent cells were counted in an area of 30 µm (width) × 80 µm (length). *P < 0.05, significant difference between wild-type and knockout mice.
exclusive, but small GTPases of the Rho family, the best-known members of which are Rac1, Rac2, cdc42, and RhoA, are prominent membrane-targeted proteins that function as critical regulators of actin cytoskeletal remodeling in neutrophils (57). Mechanical fluid stresses may also regulate the state of actin in a number of ways. Rho family GTPases are involved in cell motility induced by different mechanical stresses in endothelial and smooth muscle cells. In endothelial cells, fluid shear stress induces cytoskeletal reorganization mediated by Rac1 (52) or Rac1 and RhoA (53). The inhibition of RhoA activity with the cell-permeable C3 toxin reduces the stretch-dependent actin synthesis of vascular smooth muscle (56). The early effect of the stretch, which is associated with reduced cell polarization and directionality of smooth muscle cells, is mediated by Rac1 activity (27). In human neutrophils, osmotic pressure induces actin polymerization and cytoskeleton remodeling via activation of Rac and cdc42 (33). When differentiated HL-60 cells are subjected to physiological levels of shear stress, Rac1 and Rac2 activities are significantly decreased by shear stress in a time- and magnitude-dependent manner (Fig. 2). Because Rac induces lamellipodia formation (20), these results agree with the morphological picture of differentiated HL-60 cells under shear stress in the flow chamber because the cell area change was induced by the lamellipodia retraction. RhoA activity was transiently increased after shear stress application and returned to control level by 10 min, whereas cdc42 activity exhibited no change at any time or magnitude of shear stress (up to 5.2 dyn/cm²). Interestingly, this finding is similar to the response of endothelial cells to shear stress, although neutrophils do not make actin stress fibers. RhoA might facilitate cell detachment from the glass surface in the early stage as a regulator of uropod retraction, possibly by stimulating actomyosin filament contraction or actin depolymerization (1, 54). Cdc42 induces extension of filopodia, finger-like protrusions in the form of parallel arrays of actin filaments (20), but filopodia were rarely observed compared with lamellipodia in the flow chamber. In this study, neither a shift in cdc42 activity nor filopodia formation was observed.

GTPases function as molecular switches by cycling between an inactive GDP-bound form and an active GTP-bound form. Several proteins, such as guanine exchange factors and GTPase-activating proteins, regulate the ratio of GTP/GDP-bound forms, which determines the activities of Rho GTPases (20). Mutations that disrupt the cycling of the two forms of GTPases have been identified in the guanine nucleotide binding domain or the effector domain. Mutations at positions 12 (G12V) and 61 (Q61L) inhibit GTP hydrolysis to produce constitutively active mutants (55), and a mutation at position 17 (T17N) destabilizes the guanine nucleotide-binding site to result in a dominant negative mutant (36). Because there is no selective chemical inhibitor or activator for Rac only, these mutants are used to test the role of Rac in the signaling pathway. Both T17N Rac1 and T17N Rac2 diminished the shear stress-induced reduction of the projected cell area in the flow chamber (Fig. 3). Together with the ability of Q61LRac1 and Q61LRac2 to block the shear stress-induced reduction of cell motility, these data suggest that deactivation of Rac is required for the reduction of projected cell area in response to fluid shear stress. This result was also supported by the data with the cells taken from Rac-deficient mice (Fig. 5B).

fMLP is an effective chemoattractant for human neutrophils (4, 46). fMLP (10⁻⁹ M) also markedly increases the activities of all three Rho family small GTPases in differentiated HL-60 cells. In the presence of fMLP, the shear stress-induced Rac deactivation was completely abolished (Fig. 4A). In line with these results, fluid shear did not induce a reduction of the projected cell area after pretreatment with fMLP (Fig. 4B), suggesting again that the deactivation of Rac plays a major role in the retraction of pseudopod by physiological fluid shear stress.

Neutrophils from Rac1-null or Rac2-null mice maintain the ability to form pseudopods in response to PAF (Fig. 5A). In contrast, the response of neutrophils from both types of null mice to fMLP was weakened compared with neutrophils from the wild-type mice (16, 17, 45). Although both fMLP and PAF bind to the receptor and initiate cell migration, their signaling pathways are slightly different (3, 26, 38).

When examined in the living microcirculation, neutrophils reduced their cell length in response to fluid flow. The response was faster than the one we observed in vitro (Fig. 6), in part because the wall shear stress in vivo was higher than the one used in vitro (12.6 ± 0.7 vs. 5 dyn/cm²). Most of the leukocytes adhering to postcapillary endothelium are neutrophils with occasional monocytes (<3%), but no detectable lymphocytes, eosinophils, or basophils (37). Rac1-null neutrophils showed no change of cell length after fluid shear exposure. Fluid shear, however, produces a biphasic response in Rac2-null leukocytes. Cells that had longer pseudopods at the time of fluid shear stress application exhibited a slight decrease in cell length, whereas initially round cells showed an increase in length after shear application. The shear-induced cell response was affected by Rac1 deletion more than Rac2 deletion, an effect that might occur because mouse neutrophils have higher Rac1 than Rac2 expression, contrary to the dominant Rac2 expression in human neutrophils (28).

Fluid shear-induced retraction of neutrophil pseudopods plays an important role in the microcirculation. The retraction of pseudopods serves to minimize leukocyte entrapment in capillaries, and the circulation of leukocytes with projected pseudopods may cause significant flow reduction in capillaries (13, 50). Pseudopods also facilitate leukocyte spreading on the endothelium and may induce microvascular entrapment and capillary stasis (21, 44). We observed that Rac1-null neutrophils exhibited enhanced membrane adhesion compared with wild-type neutrophils (Table 1). The density of a microvascular network depends on a balance between angiogenesis and cell death. Adherence of neutrophils to endothelial cells is the first step of diapedesis and infiltration into the medium, where they affect the smooth muscle cells and the connective tissue with cell death and tissue alterations. Rac1-null mice showed fewer microvessels than the wild-type mice. Ti et al. (17) reported that Rac1-deficient hematopoietic stem/progenitor cells showed a significantly reduced ability to form cells. The high adherence of leukocytes and low ability for cell formation by hematopoietic stem/progenitor cells might cause less microvascular density in cremaster muscle of Rac1-null mice.

It is not clear how neutrophils decrease Rac activation in response to fluid shear. We recently reported that fluid shear serves to cleave CD18 binding sites (12), one of the integrins that can induce Rac activation. It is possible that the cleavage...
of CD18 induced by fluid shear decreases spontaneous Rac activity. Further studies are required to clarify the signaling mechanisms that cause cell retraction by fluid shear stress.

Rac has two major functions: 1) It regulates the organization of the actin cytoskeleton, and 2) it controls the activity of the NADPH oxidase enzyme complex, which is capable of producing superoxide. The lack of Rac activity may represent a risk factor for various cardiovascular complications because of the deficient leukocyte retraction in response to fluid shear in addition to unbalanced superoxide generation.

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