Platelet-Dependent Primary Hemostasis Promotes Selectin- and Integrin-Mediated Neutrophil Adhesion to Damaged Endothelium Under Flow Conditions

By P.H.M. Kuijper, H.I. Gallardo Torres, J.A.M. van der Linden, J.-W.J. Lammers, J.J. Sixma, L. Koenderman, and J.J. Zwaginga

Co-localization of blood platelets and granulocytes at sites of hemostasis and inflammation has triggered an intense interest in possible interactions between these cellular processes and induction of vessel wall injury. Leukocyte adhesion to endothelial cells decreases with increasing shear and is dependent on an initial rolling phase mediated by selectins. We hypothesized that flow-dependent platelet adhesion at an injured vessel wall will lead to P-selectin expression by platelets, thus mediating leukocyte co-localization. A perfusion chamber was used in which flowing whole blood induced platelet adhesion to a subendothelial matrix (ECM) of cultured human umbilical vein endothelial cells (HUVEC). We compared neutrophil (polymorphonuclear leukocyte [PMN]) interactions with HUVEC and their ECM with and without adhered platelets. PMNs adhered predominantly to ECM-adhered platelets and not to endothelial cells. ECM alone did not support PMN adhesion under flow conditions.

PMN adhesion to unstimulated HUVEC was only substantial at low shear (up to 200 cells/mm² at shear stress 80 mPa). In marked contrast, PMN adhesion to ECM-adhered platelets was dramatically increased, and adhesion was demonstrated at much higher shear stress (up to 640 mPa). Studies with specific antibodies showed that the platelet-dependent neutrophil adhesion was selectin-mediated. Inhibition of P-selectin caused a marked inhibition of adhesion at high shear stress, whereas the role of leukocyte L-selectin was less pronounced. β₂-integrin–blocking antibodies inhibited static neutrophil adhesion. fMLP induced L-selectin shedding from leukocytes, resulting in decreased leukocyte adhesion. In conclusion, platelet-dependent hemostasis at the ECM appears to be a powerful intermediate in neutrophil–vessel wall interactions at shear stresses that normally do not allow neutrophil adhesion to intact endothelium.

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faces was evaluated with real-time video-assisted image analysis. We show that ECM-adhered platelets are a better adhesive substrate for neutrophils, compared with cultured endothelial cells. We hypothesize that in vivo, the primary hemostatic reaction involving platelet deposition provides an intriguing mechanism for leukocyte recruitment at the damaged vessel wall.

MATERIALS AND METHODS

Monoclonal antibodies. The monoclonal antibody (MoAb) CLB/Thromb-6 (anti-β-selectin, CD62p) was kindly provided by Dr. A. Von dem Borne (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). The MoAb LAM-1-3 (anti–L-selectin, CD62L, 5 μg/mL for blocking studies) was a kind gift from T. Tedder (Harvard Medical School, Boston, MA). The MoAb WASP12.2 (anti–P-selectin, CD62p, 1 μg/mL for blocking studies) was purchased from Endogen (Boston, MA). MoAbs 44a (CD11b, 10 μg/mL for blocking studies) and IB4 (CD18, 10 μg/mL for blocking studies) were purchased from the American Type Culture Collection (Rockville, MD). The MoAb Leu-8 (anti–L-selectin, CD62L) and CSLEX1 (anti-sialylLex) were from Becton Dickinson (San Jose, CA). The MoAb 80H3 (CD66b) was purchased from Immunotech (Marseille, France).

Reagents. Percoll was obtained from Pharmacia (Uppsala, Sweden), FMLP, thrombin, and 4% formalin, 12-myracetate (PMMA) were purchased from Sigma (St Louis, MO). Experiments were performed in HEPES buffer (20 mmol/L HEPES, 132 mmol/L NaCl, 6 mmol/L KCl, 1.2 mmol/L KH₂PO₄, 1 mmol/L MgSO₄·7H₂O, 5 mmol/L glucose, and 1 mmol/L CaCl₂, pH 7.4). HEPES buffers with different free-calcium concentrations were made according to the method reported by Marks and Maxfield. The heparin Thromboliquine was obtained from Organon Teknika (Boxtel, The Netherlands). Tissue culture supplies (media, antibiotics, and trypsin) were purchased from Gibco Biocult (Paisley, UK). All other reagents were of reagent grade.

Perfusion chamber and set-up. Perfusions with steady flow were performed in a transparent rectangular perfusion chamber with a slit height of 0.3 mm and width of 6 mm. It is a modification from the original chamber described by Sakariassen et al. The chamber contains two circular plugs on which mounted cover slips are exposed to whole blood or leukocyte suspension. Cover slip surfaces were coated with either endothelial cells or ECM. Neutrophils in suspension (2 × 10⁶/mL in HEPES buffer) were aspirated from a reservoir through plastic tubing, a valve, and through the perfusion chamber with a Harvard syringe pump (Harvard Apparatus, South Natick, MA). In this way, the flow rate through the chamber could be precisely controlled. The wall shear stress (t) was calculated from the equation, t = (6Q·η)/(w·h²), in which Q is the flow rate, η is the suspending medium viscosity, w the slit width, and h is the slit height. Shear stress can be calculated in units of Pascal or as dyne per square millimeter, in which 1 Pascal is approximately 10 dynes/mm².

Endothelial cell culture. Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord veins according to the method of Jaffe et al. with some minor modifications. The cells were cultured in RPMI 1640 containing penicillin, streptomycin, and amphotericin B, with 20% (vol/vol) heat-inactivated human serum. Endothelial cells of the second passage were harvested by trypsin digestion and subcultured on glass cover slips. Endothelial monolayers used in perfusion experiments were either cultured on gelatin-coated cover slips or on cover slips upon which the isolated matrix of smooth muscle cells was present. In the latter case, HUVEC were more firmly attached and more resistant to high shear stress compared with cells grown on gelatin-coated cover slips, as described by Reutelingsperger et al. HUVEC confluency and morphology were checked by phase-contrast microscopy before and after experiments. Confluent HUVEC were used in perfusion experiments with whole blood and neutrophils. In some cases, HUVEC were stimulated with tumor necrosis factor alpha (TNF-α) before the experiment. Therefore TNF-α (100 U/mL) was diluted in fresh culture medium and incubated for 6 hours at 37°C.

Surface ECM coating. Cover slips with ECM were obtained as described previously. In short, HUVEC were cultured on 1% gelatin-coated cover slips. Cell monolayers were grown to confluence in 5 to 7 days. The matrix was obtained after removal of endothelial cells by exposure to 0.1 mol/L NH₄OH for 5 minutes at room temperature. Isolated matrices were washed and kept in phosphate-buffered saline [PBS] 1:10 vol/vol 0.1-mol/L sodium phosphate buffer in 150 mmol/L NaCl] at 4°C for a maximum of 3 weeks.

Surface platelet-covered HUVEC/ECM. Platelet adherence to different surfaces was accomplished by perfusing whole blood from healthy volunteer donors (Blood Bank, Utrecht, The Netherlands) as described previously. The blood was anticoagulated with 3,800 U/mL whole blood was perfused at 37°C for 5 minutes at a shear rate of 400 s⁻¹, which corresponds with a shear stress of approximately 2 Pa with a blood viscosity of 5 mPa·s. At this shear rate, the HUVEC monolayer stayed largely intact, while at higher shear rates HUVEC are progressively shedded from the matrix. To induce platelet adhesion to ECM-coated cover slips, 15 mL whole blood was perfused at 37°C for 7 minutes at a shear rate of 1,600 s⁻¹ (ie, a shear stress of ~7 Pa) in a recirculating system. After completion of the whole-blood perfusion, cover slips were rinsed with HEPES buffer to remove blood remnants. To determine the morphology and percentage of ECM surface covered with platelets, some cover slips were fixed (1% paraformaldehyde) and stained with May-Grünwald/Giemsa. Under given conditions, homogeneous platelet coverage of 85% ± 4% (mean ± SE, n = 24) of the surface was accomplished. The coverage was evaluated by light microscopy using a Quantimet 570C image analysis system (Leica Cambridge, Cambridge, UK).

Neutrophil isolation. Blood was obtained from healthy volunteers from the Blood Bank, Utrecht, The Netherlands. Mixed granulocytes were purified from theuffy coat of 500 mL blood anticoagulated with 0.34% (wt/vol) trisodium citrate (pH 7.4) as described previously. In short, mononuclear cells were removed by centrifugation over Ficoll-Paque (density, 1.077/mL; Pharmacia, Uppsala, Sweden). Remaining erythrocytes were lysed by incubation in isotonic 115 mmol/L ammonium chloride solution (pH 7.4) at 0°C for 20 minutes. After centrifugation, the remaining granulocytes were regenerated in RPMI 1640 (containing 0.5% human serum albumin) for 30 minutes at 37°C, diluted in HEPES buffer (2 × 10⁶/mL) and kept at room temperature until the start of the perfusion. Neutrophil purity was more than 95%, and viability measured with Trypan blue exclusion was more than 98%; morphology was controlled by light microscopy. For blocking experiments, neutrophils were preincubated with monoclonal antibodies for 30 minutes at room temperature. Before perfusion, neutrophils were always prewarmed for 5 minutes at 37°C.

Perfusion and evaluation. Neutrophil perfusions were performed as individual runs under specific shear conditions. During the perfusion, the flow chamber was mounted on a microscope stage (DM RXE; Leica, Wetzlar, Germany) equipped with a BW CCD-video camera (Sanyo, Osaka, Japan) coupled to a VHS video recorder. Perfusion experiments were recorded on video tape. Video images were evaluated for the number of adhered cells and the (rolling) velocity per cell with a Quantimet 570C image-analysis...
sion of the microscope during recording. Single adhering cells on
surface, appeared as bright white-centered cells, after proper adjust-
image analyzer. The number of surface-adhered neutrophils was
measured after 5 minutes of perfusion, unless stated otherwise, at a
minimal of 20 randomized high-power fields (total surface, ≥1
mm²). The software used was designed to automatically deter-
the (rolling) velocity of cells in focus. To accomplish this, a
sequence of a given number of images was digitally captured with a certain
time interval (Δt). At each image, the position of every cell in focus
was detected as a solid of the total area of the cell and as a central dot.
For all subsequent images, overlapping solids were added together,
resulting in a stretched total detected area for moving cells and a
round total solid area for static cells. The length and width of all
total outlines were measured, together with the total amount of dots
within each outline. Cell velocities (v) in millimeters per second
were calculated from the equation, v = [C(1 - w)]/(Δt(x - w)),
in which C is the calibration value of the microscope image-analysis
system in micrometers per pixel, l is the length and w the width of
the total outline in pixels, Δt is the time interval between images in
milliseconds, and x the number of dots within a total outline. The
cutoff value to distinguish between rolling and static adherent cells
was set at 1 μm/s. With this method, static adherent, rolling, and
freely flowing cells (which were not in focus) could be clearly distin-
guished.

Immunofluorescence flow cytometry. Surface marker expression
by activated neutrophils was determined on a FACSvantage flowcy-
tometer (Becton Dickinson Co, Mountain View, CA). Neutrophils
(2 × 10⁶/mL. HEPES buffer) were incubated with different concen-
trations of fMLP for 5 minutes at 37°C and immediately fixed in
ice-cold paraformaldehyde (1% wt/vol). The cells were stained using
anti-CD18 antibody IB4, anti-CD11b antibody 44a, anti-1–L-selectin
antibody LAM1-3, or anti-CD67 antibody, together with FITC-la-
beled goat antimouse Ig serum. The samples were kept on ice until
analysis.

Statistical analysis. Results are expressed as the mean ± SE.
Statistical analysis of the data was performed using a paired stu-
dent’s t test for single measurements or repeated-measures analysis
of variance (MANOVA) for series of measurements. P values less
than .05 were considered significant.

RESULTS
Neutrophil adhesion to a damaged vessel wall model. First, we studied the rolling and adhesion behavior of neutro-
phils on a model of a damaged vessel wall. A monolayer of
HUVEC cultured on a gelatin surface were perfused with
citrated whole blood at a shear rate of 400 s⁻¹ for 5 minutes.
This shear induced microlesions in the HUVEC monolayer
due to partial cell retraction or HUVEC shedding from their
ECM. The exposed ECM induced acute platelet adhesion,
finally covering 20% ± 5% (mean ± SE, n = 16) of the
total surface (Fig 1A). The HUVEC surface with platelet
deposition at the ECM was subsequently used to study roll-
ning and adhesive behavior of purified unstimulated neutro-
phils under flow conditions. Interestingly, neutrophils ad-
hered predominantly to blood platelets and not to endothelial
cells (Fig 1B). At a shear stress of 80 mPa, an eightfold
greater adhesion to platelet-covered surfaces as compared
with HUVEC was observed (~570 and 70 PMN/mm², re-
spectively; Table 1). At 320 mPa, this predominance of neu-
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greater adhesion to platelet-covered surfaces as compared
with HUVEC was observed (~570 and 70 PMN/mm², re-
pectively; Table 1). At 320 mPa, this predominance of neu-
triphil adhesion to platelets was even more pronounced.
Here, only 1% of adherent neutrophils interacted with HU-
VEC. Approximately half the adhering neutrophils showed a
rolling interaction with the platelet-covered spots. Rolling
neutrophils that reached the end of a platelet-rich spot often
detached from the surface.

The effect of additional stimulation of the HUVEC/plate-
let surface was examined in similar experiments in which the
surface was incubated with thrombin (1 U/mL for 5
minutes at 37°C) or TNF-α (100 U/mL for 6 hours at 37°C).
After thrombin stimulation, which induces maximal P-select-
in expression, the prevalence of the neutrophils adhering
to platelets did not change at shear stress 80 mPa, showing
a small nonsignificant decrease at 320 mPa from 97% to
95% (Table 1). TNF-α, which induces E-selectin and ICAM-
1 expression on the endothelium, did show an increased
neutrophil adhesion to HUVEC, but still more neutrophils
adhered to platelets. To further characterize the shear-depend-
tent mechanisms of neutrophil adhesion to vessel wall struc-
tures, we performed subsequent perfusion experiments in
which the reactivity of PMNs with HUVEC, ECM, or ECM-
adered platelets was separately tested.

Neutrophil adhesion to HUVEC. Neutrophil adhesion to
a HUVEC monolayer cultured on a matrix of smooth muscle
cells was examined at varying shear stresses. The observed
neutrophil adhesion was both time- and shear-dependent,
as determined by repeated-measures ANOVA (P < .01).
Neutrophil adhesion decreased with increasing shear up to
80 mPa (Fig 2); at less than 80 mPa, adhesion showed a
linear nonsaturating increase in time of up to 1,100 cells/
mm² after 20 minutes. At a shear stress of 200 mPa (2 dynes/
mm²) and higher, no significant PMN rolling or adhesion to
HUVEC could be detected. Incubation of HUVEC with
TNF-α (100 U/mL for 6 hours at 37°C) increased neutrophil
adhesion at all shear stresses, allowing adhesion up to a
shear of 320 mPa (Fig 2).

To examine the effect of stimulus-induced maximal P-
selectin expression, HUVEC were stimulated with thrombin
(1 U/mL for 5 minutes) before PMN perfusion.25 Table 2
shows the increased neutrophil adhesion at shear stress 40
mPa to stimulated HUVEC versus unstimulated HUVEC.
After the experiments, HUVEC did not show shear-induced
changes in morphology, as examined with a phase-contrast
microscope. Thrombin-stimulated HUVEC were slightly
contracted after perfusion, but still covered 80% to 90% of
the surface. Higher thrombin concentrations showed no
additional effect on PMN adhesion but increased the change
in HUVEC morphology (data not shown). Prestimulation of
neutrophils with the chemotaxin fMLP at a concentration of
10⁻⁷ mol/L, which is known to induce integrin activation,27
resulted in increased PMN adhesion (Table 2). Stimulation
of neutrophils with fMLP caused pseudopodia formation in
40% to 50% of the PMNs. Total adhesion was increased
more after simultaneous stimulation of both neutrophils and
HUVEC. These results are largely in agreement with known
characteristics of neutrophil-EC interactions,6,28,29 thus val-
idating the perfusion system.

Neutrophil adhesion to ECM. HUVEC ECM alone did
not support PMN adhesion under flow conditions (shear
A monolayer of endothelial cells was perfused for 2 minutes with citrated whole blood at a shear rate of 400 s⁻². The surface is covered with spread, slightly contracted HUVEC and platelets adhering to ECM (arrows). Unstimulated neutrophils adhere predominantly to the platelet-covered loci under flow conditions (arrows). Immunofluorescent staining of ECM-bound platelets using a P-selectin antibody. Unstimulated neutrophils adhered to the platelets bound to ECM (arrow) and not to ECM alone (*) under flow conditions.

Neutrophil adhesion to ECM-bound platelets. Since we have observed that activated platelets were more potent in supporting neutrophil adhesion in flow as compared with (activated) endothelium, we examined the platelet-PMN interaction in more detail. Platelets were allowed to adhere to ECM during a perfusion with anticoagulated whole blood (7 minutes at shear rate 1,600 s⁻¹). The platelets covered 85% ± 3% (mean ± SE, n = 24) of the ECM surface and were spread or dendritic with a few small aggregates. Immunofluorescent staining of ECM-adhered platelets with a...
PMNs ADHERE TO PLATELETS UNDER FLOW CONDITIONS

Table 1. Neutrophil Adhesion to Endothelial Cells and ECM-Bound Platelets Under Flow: Effects of Shear Stress and Stimulation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>+ Thrombin</th>
<th>+ TNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells/mm²</td>
<td>80 mPa</td>
<td>320 mPa</td>
<td></td>
</tr>
<tr>
<td>PMNs/mm² adhered to platelets: HUVEC</td>
<td>570:70</td>
<td>570:410</td>
<td></td>
</tr>
<tr>
<td>% Neutrophil adhesion to platelets*</td>
<td>97</td>
<td>85</td>
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</table>

A monolayer of HUVEC cultured on a gelatin surface were perfused with citrated whole blood at a shear rate of 400 s⁻¹ for 5 minutes. A second perfusion with neutrophils (10⁶ cells/mL) was performed at a shear stress of 80 or 320 mPa for 5 minutes (see Fig 1A and B). Thrombin (1 U/mL) was used to stimulate HUVEC and blood platelets on a coverslip for 5 minutes before the neutrophil perfusion. HUVEC were incubated with TNF (100 U/mL) for 6 hours at 37°C and washed before the whole blood perfusion. Platelet coverage and neutrophil adhesion to either platelets or HUVEC were determined using an image analyzer system.

Table 2. Effect of Stimulation on Neutrophil Adhesion to a HUVEC Monolayer Under Flow Conditions

<table>
<thead>
<tr>
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<th>Resting HUVEC</th>
<th>HUVEC + Thrombin 1 U/mL</th>
</tr>
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<tbody>
<tr>
<td>Neutrophils + fMLP 10⁻⁷ mol/L</td>
<td>60 ± 20*</td>
<td>401 ± 60*</td>
</tr>
<tr>
<td>Neutrophils +</td>
<td>350 ± 60*</td>
<td>572 ± 60</td>
</tr>
</tbody>
</table>

Neutrophils were perfused over a HUVEC monolayer at shear stress 40 mPa with or without stimulation. Neutrophils (2 × 10⁶/mL HEPES buffer) were stimulated with chemotaxin fMLP (10⁻⁷ mol/L) 5 minutes before perfusion at 37°C. HUVEC were activated 5 minutes before perfusion with thrombin (1 U/mL HEPES), and thrombin was washed away before perfusion. The number of surface-interacting neutrophils per mm² after 5 minutes perfusion was represented as the mean ± SE of 3 experiments.

* Statistically significant difference between two perfusions with unstimulated and stimulated cells determined by Student’s two-tailed t-test (P < .05).
Fig 3. Neutrophil rolling and adhesion to ECM-bound platelets: effect of shear stress. (A) Number of neutrophils adhering under flow conditions to ECM-coated cover slips with or without preadhered platelets. After a first perfusion with anticoagulated whole blood (○), platelet poor plasma (□), or filtered plasma (△) over an ECM-coated cover slip, the surface was covered with 85%, <2%, or 0% spread platelets, respectively. Subsequently, the total number of adhering and rolling neutrophils/mm² were measured after 5 minutes of perfusion. Mean ± SE of 6 experiments shown. (B) Rolling velocity and percentage of rolling neutrophils during perfusion at different shear stresses. An ECM-coated cover slip preperfused with anticoagulated whole blood and therefore covered with platelets (85% of the surface) was perfused with resting neutrophils (2 x 10⁷/mL HEPES buffer) at a shear stress varying from 20 to 680 mPa. (□) Number of rolling neutrophils as a percentage of total adhered cells; (△) Rolling velocity (μm/sec). Mean ± SE of 6 experiments shown.

Fig 4. Effect of calcium depletion and heparin on neutrophil adhesion to ECM-bound platelets. Neutrophils were perfused over a surface of ECM-bound platelets as described earlier. After 5 minutes, the total number of adhering neutrophils was calculated as neutrophils/mm². Numbers are given as % of control values. (A) Neutrophils were preincubated in HEPES buffer with different concentrations of free Ca²⁺ in the presence of 1 mmol/L Mg²⁺ for 5 minutes at 37°C. Subsequently, perfusions were performed at shear stress 90 mPa (■) and 320 mPa (△). Mean ± SE of 3 experiments shown. Significant effects of Ca²⁺ depletion and different shear stresses were determined by repeated-measures ANOVA (P < .05). (B) Neutrophils were incubated with different concentrations of heparin (thrombolipin) for 5 minutes at 37°C; perfusions were performed at shear stress 90 mPa (■) and shear stress 320 mPa (△). Mean ± SE of 4 to 6 experiments shown. Significant effects of heparin incubation and different shear stresses are determined by repeated-measures ANOVA (P < .05).
PMNs ADHERE TO PLATELETS UNDER FLOW CONDITIONS

Table 3. Neutrophil Adhesion to ECM-Bound Platelets Under Flow: Effects of Monoclonal Antibodies

<table>
<thead>
<tr>
<th></th>
<th>80 mPa (cells/mm²)</th>
<th>320 mPa (cells/mm²)</th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Rolling</td>
</tr>
<tr>
<td>Control</td>
<td>963 ± 131</td>
<td>190 ± 42</td>
</tr>
<tr>
<td>D6</td>
<td>1,002 ± 170</td>
<td>183 ± 65</td>
</tr>
<tr>
<td>CD18</td>
<td>816 ± 130</td>
<td>360 ± 69*</td>
</tr>
<tr>
<td>CD11b</td>
<td>873 ± 78</td>
<td>408 ± 79*</td>
</tr>
<tr>
<td>L-sel</td>
<td>752 ± 120</td>
<td>140 ± 28</td>
</tr>
<tr>
<td>P-sel</td>
<td>368 ± 45*</td>
<td>111 ± 44</td>
</tr>
<tr>
<td>P-sel/L-sel</td>
<td>294 ± 87*</td>
<td>12 ± 8*†</td>
</tr>
<tr>
<td>P-sel/CD18</td>
<td>247 ± 44*</td>
<td>ND</td>
</tr>
<tr>
<td>P-sel/L-sel/CD18</td>
<td>52 ± 16†</td>
<td>ND</td>
</tr>
</tbody>
</table>

Cover slips with ECM-bound platelets were incubated with control HEPES buffer or with anti-P-selectin (Waps 12.2); neutrophils were incubated with control HEPES buffer, anti-L-selectin (LAM1-3), anti-CD18 (IB4), and/or anti-CD11b (44a) for 30 minutes at room temperature. Neutrophils (2 × 10⁶/mL HEPES buffer) were perfused in the presence of the monoclonal antibodies at 37°C at shear stresses of 80 and 320 mPa. Data represent the total number of adhering and rolling neutrophils per mm², or the number of rolling cells per mm² after 5 minutes of perfusion (mean ± SE, n = 4-6).

Abbreviation: ND, not determined.

* Statistically significant effect of antibody treatment vs control cells (P < .05).
† Statistically significant decrease vs P-selectin–treated cells (P < .05).

neutrophils before perfusion caused a decrease in adhesion in a concentration- and shear-dependent manner. At shear stress 320 mPa, addition of 0.1 U/mL heparin already showed an inhibition of 35% (Fig 4B). Under these conditions, absolute inhibition of 97% was seen at a concentration of 250 U/mL heparin, whereas at 80 mPa, inhibition was significantly less effective (63%).

Inhibition of neutrophil-platelet interaction by antibodies. The role of P- and L-selectin and β₂-integrins in neutrophil adhesion to platelets was investigated using monoclonal antibodies. We determined the role of P-selectin expressed by platelets by incubating the platelet surface with monoclonal antibody WASP12.2 against P-selectin (15 minutes at RT). Subsequent perfusion with PMNs over the P-selectin–blocked platelet surface showed a 60% decrease at shear stress 80 mPa, whereas at 320 mPa, approximately 90% of neutrophil adhesion was inhibited (Table 3). Further studies were performed by blocking L-selectin by preincubation of the neutrophils with the antibody LAM1-3. This resulted in a significant inhibition of 70% of neutrophil adhesion at shear stress 320 mPa (P < .05), whereas no significant inhibition was seen at lower shear (Table 3). Blocking of β₂-integrins with the CD11b (clone 44a) and CD18 antibodies (clone IB4) did show a small but nonsignificant inhibition of the total number of PMNs adhered to the surface at shear stresses of 80 and 320 mPa (Table 3). However, at 80 mPa, the number of rolling cells increased significantly compared with the control level (183 rolling PMNs/mm² with control monoclonal antibody, compared with 360 and 408 rolling PMNs/mm² using CD18 and CD11b, respectively). The functional blocking capacity of the integrin antibodies was confirmed in a neutrophil aggregation assay. Here, the antibodies effectively inhibited PMA-stimulated aggregation, which is integrin-mediated (results not shown). Functional inhibition of both P- and L-selectins resulted in a decrease in the number of rolling cells. Interestingly, at the shear stress of 80 mPa, total inhibition (>95%) of neutrophil adhesion was only observed when P-selectin, L-selectin, and CD18 antibodies were used simultaneously. The results indicate that the integrin antibodies can only block static adhesion, whereas the selectin antibodies are more effective in blocking the rolling interaction of PMNs to platelets. In all experiments, similar incubations with isotype-matched control antibodies showed no significant effects.

Neutrophil adhesion to platelets after fMLP stimulation. To characterize the effect of neutrophil stimulation on PMN interaction with ECM-associated platelets, we incubated PMNs with the bacterial formyl peptide and chemoattractant, fMLP. Subsequent perfusions were performed at different shear stresses. In contrast to the increased adhesion by activation of neutrophils to HUVEC (Table 2), fMLP stimulation of PMNs caused a shear-dependent inhibition of neutrophil adhesion to platelets (Fig 5A). At shear stress 20 and 40 mPa, no significant inhibition was observed. At shear stress 80 and 320 mPa, activation with fMLP (10⁻⁷ mol/L) resulted in a maximal inhibition of 50%. The absolute number of rolling neutrophils per square millimeter after activation with fMLP was also determined (Fig 5B).

Neutrophil rolling was decreased dose-dependently after stimulation with fMLP at both shear stresses, with a maximal inhibition of 70% after stimulation with 10⁻⁷ mol/L fMLP. A more pronounced effect of fMLP treatment was observed at a shear stress of 80 mPa versus 320 mPa. We measured the expression of L-selectin, CD11b, CD18, and a neutrophil degranulation/activation marker (CD67) to determine the effect of fMLP treatment on the expression of adhesion molecules by neutrophils. L-selectin expression at the neutrophil surface was decreased by 60% upon stimulation with fMLP at concentrations of 10⁻⁸ mol/L and higher. Under the same conditions, CD11b and CD67 expression increased by 90% and 65%, respectively, while CD18 expression was unaffected (results not shown).

DISCUSSION

Neutrophil adhesion to HUVEC. In our experiments, we used a well-defined perfusion system to study neutrophil interaction with ECM-bound platelets, and to compare this interaction with adhesion of PMNs to HUVEC. The initial attachment of leukocytes to endothelial cells under flow conditions is known to be mediated by a selectin-dependent rolling interaction. Subsequent integrin binding is supposed to be necessary for static adhesion, spreading, and transmigration. In accordance with these studies, we observed diminished neutrophil adhesion to unstimulated HUVEC with increasing shear and virtually no adhesion at a shear stress of 200 mPa or higher (Fig 2). We studied the effect of induction of adhesive properties of both endothelial...
Neutrophil adhesion and rolling to ECM-bound platelets: effect of fMLP stimulation. Neutrophils were incubated with different concentrations of fMLP for 5 minutes at 37°C and perfused over a surface of ECM-bound platelets. After 5 minutes, the number of adhered and rolling neutrophils was calculated as neutrophils/mm². (A) Effect of fMLP stimulation on the total number of adhered neutrophils. Perforusions were performed at shear stress 20 mPa (△), 40 mPa (▼), 80 mPa (●), and 320 mPa (▲). Data represent the total number of adhered cells (sum of static adhered and rolling cells) per mm² as a percentage of the unstimulated control perfusion (mean ± SE of 4 to 7 experiments). A statistically significant effect of different shear stresses was determined by ANOVA (Pi < 0.05). (B) Effect of fMLP stimulation on the number of rolling neutrophils. Perforusions were performed at shear stress 80 mPa (●) and 320 mPa (▲). Data represent the number of rolling cells per mm² (mean ± SE of 4 to 6 experiments). Statistically significant effect of fMLP treatment and of different shear stresses was determined by repeated-measures ANOVA (P < 0.02).

Neutrophil adhesion to platelets. The platelet surface we used was formed on the matrix of endothelium in the presence of whole blood and under physiologic flow conditions. Subsequent perforusions showed that high numbers of neutrophils (up to 1,600/mm²) adhered to these platelet surfaces (Fig 3A). The absence of PMN adhesion to bare or plasma-perfused ECM clearly indicated the platelet-dependency. Both the mean rolling velocity and the percentage of rolling neutrophils increased with higher shear (Fig 3B). Interestingly, the maximal percentage of rolling cells does not exceed 60%, indicating that even at a high shear stress, part of the neutrophils are able to adhere firmly to the platelet-covered surface. Moreover, a maximal rolling velocity was observed, probably related to the number of available rolling receptors. ECM-adherent platelets seem to support more PMN adhesion at higher shear stresses as compared with HUVEC (compare Figs 3A and 2). This was nicely illustrated in perforusions in which both HUVEC and ECM-adhered platelets were present (Fig 1 and Table 1).

Selectins and ligands. Receptors and ligands involved in the adhesion of neutrophils to platelets under flow were further characterized. Clearly, neutrophil adhesion to ECM-bound and activated platelets under flow was critically dependent on selectins, particularly at higher shear stresses (Table 3 and Fig 4B). Earlier reports have shown that P-selectin supported adhesion of thrombin-activated platelets in suspension to neutrophils. Moreover, in vivo studies have shown that antibodies against P- and L-selectin have a protective effect against an experimentally induced inflammatory reaction and pulmonary vascular damage in animal models. The fact that heparin treatment, known to pre-
vent selectin-ligand binding in vitro and during acute inflammation in vivo.\(^4\)\(^3\)\(^4\) induced shear-dependent inhibition was in agreement with the role for selectin/mucin-ligand interactions. At high shear, adhesion was blocked more than 90% by anti-P-selectin. Also, anti-L-selectin was partially able to inhibit PMN adhesion at high shear stress (Table 3). This could be an indication for a possible interaction between P- and L-selectin. L-selectin does indeed possess the sLeX carbohydrates that may serve as a ligand for P-selectin.\(^4\)\(^3\)\(^4\) Nevertheless, P-selectin recognizes other ligands, such as PMN-associated P-selectin glycoprotein ligand-1 (PSGL-1), as well.\(^4\)\(^3\)\(^4\) An alternative (mucin-like) ligand for L-selectin that could be present on the platelet surface remains to be defined; neuraminidase treatment of the platelet surface decreased neutrophil rolling at shear 80 mPa by approximately 50% and the total number of adhered neutrophils by approximately 30% (data not shown). Additional evidence for the role of L-selectin may be deduced from the fact that fMLP-treated neutrophils adhered less to the platelet surface at shear stresses of 80 and 320 mPa (Fig 5A). The concentration of fMLP that induced the inhibitory effect also caused \(\beta_2\) integrin activation and L-selectin shedding from the neutrophil surface (as confirmed by FACS experiments). Under high-shear conditions, which favor selectin-mediated adhesion, shedding of L-selectin\(^2\) obviously overshadows a simultaneous increase of integrin-dependent adhesion. In contrast, leukocyte adhesion to unstimulated HUVEC, which is integrin-dependent and only occurs at low shear, is enhanced after stimulation with fMLP\(^28\)\(^29\) (Fig 2). In the experiments with HUVEC, the presence of L-selectin seems less critical for leukocyte adhesion.

**Role of integrins.** Indirect evidence for the role of integrin-dependent adhesion of PMNs to ECM-bound platelets could be derived from the fMLP experiments. Preactivated neutrophils exhibited 70% less rolling and more static adhesion as compared with unstimulated neutrophils (Fig 5B). At a low shear stress (25 mPa), when integrin-mediated adhesion is less dependent on rolling, fMLP prestimulation tends to increase PMN adhesion. In agreement, at shear 320 mPa, addition of fMLP (10\(^{-8}\) mol/L) during the perfusion resulted in an immediate arrest of all rolling cells and subsequent spreading (results not shown). Our results indicate that rolling facilitated integrin-mediated adhesion. In agreement with the findings obtained by Buttrum et al.\(^1\) both CD18 and CD11b antibodies failed to significantly decrease the total amount of neutrophils adhered to ECM-bound platelets. However, we observed that these monoclonal antibodies caused a clear shift toward rolling adhesion at shear stress 80 mPa (Table 3). When both P- and L-selectins were blocked with monoclonal antibodies, some neutrophil adhesion could still be observed, which was abolished when CD18 was added. The data on fMLP-induced and integrin monoclonal antibody–blocked static adhesion (Table 3) indicated a clear role for integrins in these processes.

**Integrins and ligands.** The finding that calcium depletion during perfusion completely inhibits PMN adhesion to platelets (Fig 4A) demonstrated the calcium dependency of the process, and is in agreement with the described calcium-dependent binding of selectins and integrins to their ligands.\(^4\)\(^3\)\(^4\) In studies by others, in which isolated platelets adhered to lysine-coated glass under static conditions, the characteristics of PMN adhesion were different.\(^6\)\(^3\)\(^7\) In contrast to our results, PMNs adhered only at shear stresses less than 200 mPa, and no integrin-dependent adhesion was reported. Additionally, in these studies, 60% of the cells show rolling adhesion at 80 mPa, as compared with 15% in our experiments. The described differences could be explained by the presence of more or more potent integrin ligands in our system. The use of extracellular matrix and flowing whole blood to prepare the platelet surface not only may change the platelet activation during adhesion but could also influence the presence of plasma proteins on the surface. A possible ligand for the \(\beta_2\)-integrins in our system is ICAM-2, which has been reported to be expressed on platelets.\(^4\)\(^7\)**\(^4\)\(^7\)** However, preliminary experiments show that incubation of the platelet surface with ICAM-2 antibodies only slightly inhibited neutrophil adhesion (15% \(\pm\) 7%, \(n = 3\)). Fibrogen, which is present in high concentrations during the whole-blood perfusion, is a second candidate for \(\beta_2\)-integrin–mediated adhesion. It binds activated platelets and may become the ligand for \(\beta_2\)-integrins on neutrophils.\(^4\)\(^8\)\(^4\)\(^9\) However, the precise role of integrin binding and activation in PMN-platelet interactions under flow conditions remains to be characterized.

In conclusion, our model for endothelial damage provides evidence that activated platelets are more potent in supporting neutrophil adhesion under flow as compared with (activated) endothelium, particularly at higher shear stresses. An explanation for our findings may be that matrix-activated platelets express P-selectin in much higher numbers per surface area and for a longer period as compared with thrombin-activated HUVEC.\(^4\)\(^2\)\(^3\) Long-term activation of the endothelium (eg, by TNF) can further increase PMN adhesion by upregulation of ICAM-1 and E-selectin, but adhesion to activated platelets remains more potent. PMN adhesion to the platelet surface can be characterized by a rolling interaction, which is dependent on selectins, and a static adhesion. The described mechanisms are likely of key importance in the pathogenesis of thrombosis, atherosclerosis, or vasculitis, in which the inflammatory response goes hand in hand with platelet-dependent hemostasis.

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