Minimal Platelet Deposition and Activation in Models of Injured Vessel Wall Ensure Optimal Neutrophil Adhesion Under Flow Conditions

J.J. Zwaginga, H.I. Gallardo Torres, J.-W.J. Lammers, J.J. Sixma, L. Koenderman, P.H.M. Kuijper

Abstract—Platelets at injured vessel wall form an adhesive surface for leukocyte adhesion. The precise relation between platelet adhesion and activation and leukocyte adhesion, however, is not known. We therefore used various models of injured vessel wall to form different patterns of platelet adhesion. The interaction of polymorphonuclear neutrophils (PMNs) was subsequently studied under flow conditions. In the absence of platelets, not only endothelial cell, smooth muscle cell, and fibroblast matrices but also purified matrix proteins (fibrinogen, collagen, and fibronectin) barely support PMN adhesion. The presence of platelets, however, strongly enhances PMN adhesion. PMN adhesion shows a proportional increase with platelet coverage up to 15%. Although PMNs roll over the scarcely scattered platelets, they speed up again when encountering surfaces without platelets. This “hopping” interaction of PMNs vanishes with platelet coverage >15%. Unobstructed rolling of PMNs is then observed and soon leads to a maximal adhesion of 1000 to 1200 cells/mm². The mean rolling velocity of PMNs continues to decrease with higher platelet coverage. Platelet aggregate formation is an accepted indicator of platelet activation. The presence of platelet aggregates instead of contact or spread platelets, however, does not increase PMN adhesion. Also, additional stimulation of surface-associated platelets by thrombin fails to influence PMN adhesion. Moreover, indomethacin as an inhibitor of platelet activation and aggregation does not change the subsequent PMN interaction. In conclusion, approximately 15% of platelet coverage is sufficient for optimal PMN adhesion. Increasing platelet coverage increases the availability of platelet-associated receptors that lower PMN rolling velocity. Additional activation of adherent platelets makes no difference in the expression of relevant adhesion receptors. Therefore, minimal vascular damage in vivo and only scarce platelet adhesion will already evoke significant colocalization of leukocytes. (Arterioscler Thromb Vasc Biol. 1999;19:1549-1554.)

Key Words: platelet adhesiveness ■ thrombosis pathophysiology ■ vascular injury ■ leukocyte adhesion ■ flow

Platelet adhesion at the injured vessel wall was convincingly shown to support leukocyte adhesion under flow conditions.1–3 The observed colocalization of the hemostatic and inflammatory response in thrombosis, vasculitis, and atherosclerosis in vivo supports the role of these interactions in pathophysiology of these diseases.4–6 Recent research both in vivo and in vitro in perfusion chambers showed that platelets at the injured vessel wall form an extremely powerful substrate for leukocyte, eg, polymorphonuclear neutrophil (PMN) adhesion under flow conditions. The first step in PMN adhesion consists of a rolling or tethering interaction involving P-selectin1–3 expressed by activated platelets and P-selectin glycoprotein ligand-1 by leukocytes. Firm adhesion follows by integrin–ligand interactions. Mac-1 (CD11bCD18) was identified to be the main leukocyte β2 integrin that binds to the platelet surface. Intercellular adhesion molecule-2 (ICAM-2) (at platelets) or fibrin(ogen) embedded in the thrombus or associated with platelet glycoprotein IIbIIIa are main ligands for Mac-17–9. Moreover, subendothelium contains several ligands for both β2 and β1 integrins expressed by leukocytes.10–13 These subendothelial matrix proteins, however, are insufficient for shear-resistant attachment of leukocytes under flow conditions. Therefore, PMN adhesion under flow conditions remains dependent on initial selectin-mediated tethering or rolling. The presence of platelet-associated P-selectin at the injured vessel wall without endothelium is critical for the subsequent inflammatory response.

Up to now, platelet-dependent PMN adherence was studied at surfaces that were almost completely covered with spread platelets. Normal platelet adhesion, however, varies not only in extent of coverage, but also in morphology. Initial platelet adhesion is characterized by often reversible adhesion of spherical (contact) platelets that readily spread out on the surface. The formation of platelet aggregates is a strong indicator of platelet activation and is regulated not only by the

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From the Departments of Hematology (J.J.Z., J.J.S.) and Pulmonary Diseases (H.I.G.T., J.-W.J.L., L.K., P.H.M.K.), University Hospital Utrecht, Netherlands
Correspondence to J.J. Zwaginga, MD, PhD, Department of Hematology, University Hospital Utrecht, Heidelberglaan 100, PO BOX 85500, 3584 CX, Utrecht, the Netherlands. E-mail J.J.Zwaginga@digd.azu.nl
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protein composition of the subendothelial tissue but also by local stimuli such as coagulation-dependent thrombin and platelet-secreted stimuli. The influence of platelet coverage, platelet adhesion morphology, and the related activation state of these platelets on PMN adhesion is unknown and is the subject of our study. We hypothesized that an increase in platelet coverage and aggregate formation or activation would enhance platelet-dependent PMN adhesion. Surprisingly, our results seem to indicate that even a low coverage of nonaggregated platelets ensures optimal leukocyte adhesion.

Methods

Reagents
Ficoll was obtained from Pharmacia. Tissue culture supplies (media, antibiotics, and trypsin) were purchased from Gibco Biocult. Fibrinogen was obtained from Enzyme Research Laboratory, collagen was obtained from Nycomed, and human albumin was obtained from Behringwerke. All other reagents were of reagent grade.

Vessel Wall Models and Perfusion Surfaces
All cells were of human origin. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins with minor modifications. Endothelial cells of the second passage were harvested by trypsin digestion and subcultured on formaldehyde-fixed gelatin (1%)-coated glass coverslips. Smooth muscle cells (SMCs) were isolated from human umbilical arteries as described before. Fibroblasts were isolated from pulmonary arteries. The HUVECs and SMCs were cultured in RPMI 1640 containing penicillin, streptomycin, and amphotericin B with 20% (vol/vol) heat-inactivated normal human serum. The medium for fibroblasts contained 10% pooled bovine serum instead. HUVECs reached confluence in 5 to 7 days, SMCs and fibroblasts reached confluence within 5 to 12 days.

Endothelial cell matrix (ECM), fibroblast matrix (FM), and smooth muscle cell matrix (SCM) were isolated by removing the confluent cells by treatment with 0.1 mol/L NH₄ OH (5 to 15 minutes at room temperature) and subsequently keeping the treated matrices in PBS at 4°C for a maximum of 2 to 3 weeks. Fibrinogen was coated on gelatin-coated glass coverslips (1% wt/wt gelatin in PBS, 10 minutes at room temperature) by incubation with fibrinogen (1 mg/mL) in PBS for 30 minutes at room temperature. Collagen (1 mg/mL) was coated the day before use with the aid of a retouching air brush at a nitrogen pressure of 1 atm. The collagen was sprayed at a density of approximately 30 µg/cm².

Fibronectin was purified as described by Houdijk et al. Coated on gelatin-coated glass coverslips by incubation with fibronectin (Fn; 100 µg/mL) in PBS, and used the same day. All coated coverslips were blocked for specific binding with a 1% human albumin solution in PBS.

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 anticoagulated with 0.34% (wt/vol) trisodiumcitrate (pH 7.4) as described before. PMN purity was >95% with a viability (trypan-blue exclusion method) of >98%; PMN morphology was checked by light microscopy. Maximal allowed presence of polarized aggregated cells was 1%. Isolated PMNs were suspended in HEPES buffer at 2 × 10⁶/mL and kept at room temperature until the start of perfusion.

Perfusion Chamber and Set-Up
Perfusion with laminar nonpulsatile flow was performed in a transparent rectangular perfusion chamber with a slit height of 0.3 mm and width of 6 mm. The chamber is a modification from the original. The chamber holds 2 circular knobs on which vessel wall-model-containing coverslips can be mounted; in this way the vessel walls are exposed to whole blood or leukocyte suspensions. Perfusion with laminar nonpulsatile flow was performed in a transparent rectangular perfusion chamber with a slit height of 0.3 mm and width of 6 mm. The chamber is a modification from the original. The chamber holds 2 circular knobs on which vessel wall-model-containing coverslips can be mounted; in this way the vessel walls are exposed to whole blood or leukocyte suspensions. Perfu...
platelet adhesion of mainly spread platelets and some small aggregates between 7.5% and complete (>95%) coverage. As a control for no platelet coverage (<2%) we perfused the ECM with platelet-poor plasma. In subsequent perfusions with isolated and resting PMNs (at 250 s⁻¹=2 dynes/cm²) total adhesion, percentage of rolling PMNs, and rolling velocity were evaluated and correlated with the platelet coverage. The latter 2 parameters could only be measured at >15% platelet coverage. Below this coverage, we observed PMNs to stick, roll, and detach between the sparsely present platelets on the surface. This "hopping" behavior, which can also be described as a sort of tethering, interfered with the computer-assisted evaluation. Secondary tethering (PMNs rolling over PMNs already associated with the surface, leading to cluster formation) was also observed but was not different under the various conditions.

PMN adhesion (Figure 1) clearly increases with platelet coverage of ECM (logarithmic fit with coefficients y₀=349, a₁=230; R=0.73; P<0.01). The PMN adhesion at platelet coverage >15% soon reaches a maximum of 1000 to 1200 cells/mm². In contrast with PMN adhesion, PMN rolling velocity (Figure 2) decreased with increasing platelet coverage from 12 μm/s at 20% platelet coverage to 4 μm/s at 95% platelet coverage (correlation coefficient, −0.63; P<0.01). This decrease in rolling velocity underlines that greater selectin density increases the possible interactions of PMNs with the platelet-covered ECM. Although the percentage of PMNs that rolled on platelets was less at 95% platelet coverage (12%) than at 20% platelet coverage (22%), no significant correlation between both parameters was reached (correlation coefficient, −0.08; P>0.1) (Figure 3).

PMN Adherence to FbM and SMCM: Effect of Local Platelet Presence

FbM and SMCM, as models for different depths of vessel wall injury, were perfused with whole blood. ECM perfusions were included as a control. To obtain comparable platelet coverage at the different surfaces we perfused ECM for 2 minutes and FbM and SMCM both for 5 minutes. Subsequent PMN adhesion to these surfaces was studied in the above-mentioned manner. Again, prior perfusion with platelet-poor plasma was used to study the PMN adherence to ECM, FbM, and SMCM that was not dependent on platelet presence.

Platelet adhesion at the surfaces was comparable in coverage (approximately 30%) and morphology, consisting predominantly of spread platelets and some small aggregates. Total PMN adhesion to the platelet-covered surfaces showed large variations (600 to 1100 cells/mm²; Table 1) but no significant differences were observed. Platelet-covered FbM, however, was more effective in slowing down PMNs.

**Figure 1.** PMN adhesion at different amounts of platelet coverage on ECM. The varying amounts of platelet coverage were obtained by whole blood perfusion at 1600 s⁻¹ ranging from 1 to 5 minutes in 5 different experiments. The ECM-associated platelets were subsequently perfused with PMN at 250 s⁻¹ (±2 dynes/cm²) for 5 minutes. Total PMN adhesion was evaluated. A logarithmic fit was calculated and depicted: y=349+230×ln(x); R=0.73; P<0.01.

**Figure 2.** PMN rolling velocity was evaluated at different platelet coverage on ECM (for description, see Figure 1). An exponential fit was calculated and depicted: y=74.9×x⁻⁰.⁶³, P<0.01.

**Figure 3.** The percentage of rolling cells on the total of adherent cells (the sum of rolling and firmly adherent cells) was evaluated at the different platelet coverage on ECM (for description, see Figure 1). A linear fit is depicted: y=23.7−0.087×x; P>0.1.

<table>
<thead>
<tr>
<th>TABLE 1. PMN Adhesion, Percentage of Rolling Cells, and Rolling Velocity at Platelets Associated With ECM, FbM, and SMCM</th>
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<td><strong>Platelet</strong></td>
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<td><strong>Coverage,</strong></td>
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<td>%</td>
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<tr>
<td>ECM</td>
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<td>ECM</td>
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<td>FbM</td>
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<td>FbM</td>
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NE indicates not evaluable.

*Indicates P<0.05.
(P<0.05) and tended to be more effective in supporting total adhesion than ECM with platelets. PMN adhesion to naked surfaces was <15% of the PMN adhesion that was present at platelet-covered matrices, indicating the clear dependency of PMN adhesion for surface-associated platelets.

**PMN Adhesion to Platelets at Purified Matrix Proteins**

The protein composition of vessel wall models determines not only the platelet coverage but also the activation and morphology of the adhering platelets. We further tested whether specific components of the vessel wall change platelet adhesion and activation enough to also influence subsequent PMN adhesion. Fibrinogen, collagen type III, and Fn were first exposed to whole blood perfusion to obtain typical platelet adherence. Protein surfaces without platelet adhesion were prepared by perfusing with platelet-poor plasma.

On Fn, total platelet coverage was low (5%), even with longer (10 minutes) whole blood perfusion. On collagen and fibrinogen, however, platelet coverage was comparable to that with ECM, and well above 15% platelet coverage in this sense was clearly surface-dependent. Furthermore, platelet morphology differed considerably among the surfaces. On ECM, Fn, and fibrinogen, spread platelets predominated, whereas collagen induced clear formation of platelet aggregates. Subsequent PMN adhesion to these platelet-covered surfaces was evaluated and compared with adhesion to platelets on ECM in parallel experiments (Table 2).

In accordance with the results obtained for low platelet coverage on ECM, the low platelet coverage on Fn resulted in less PMN adhesion. Moreover, Fn itself without platelets showed less PMN adhesion than the other surfaces. The predominantly spread platelets on fibrinogen supported PMN adhesion equally as well as ECM with platelets. The aggregates on collagen did not affect PMN adhesion either. The rolling velocity and the percentage of PMN that rolled on collagen-, fibrinogen-, or ECM-associated platelets did not differ in these experiments. On Fn, the latter parameters could not be obtained because of low platelet coverage and low PMN adhesion.

**PMN Adherence to Platelets With Different Morphology: Effect of Additional Platelet Stimulation or Inhibition**

Additional stimuli such as thrombin generated by the coagulation pathway or stimuli secreted by activated platelets cause platelet aggregate and thus thrombus formation. Most platelet inhibitors are designed to inhibit platelet aggregation. We investigated whether PMN adhesion was influenced by the presence of platelet aggregates instead of spread platelets. Moreover, we tested whether additional activation or inhibition of surface-associated platelets changed the PMN adhesion. For this purpose, collagen-coated coverslips were perfused with whole blood with and without indomethacin. In this way normal platelet-coated coverslips were used as a control. Additional stimulation of surface-adherent platelets was obtained by thrombin (1 U/mL) treatment. Platelet coverage on ECM and collagen was comparable (approximately 60% to 70%); addition of indomethacin to the perfusate or thrombin stimulation of the adherent platelets did not change the amount of platelet coverage. On collagen, the flow-oriented aggregate formation was adequately inhibited by indomethacin (Figure 4). The predominantly spread platelet adhesion on ECM was minimally influenced by indomethacin, but formation of small aggregates was inhibited. Thrombin treatment flattened out the contact platelets and aggregates present at both surfaces.

Notwithstanding the clear differences in platelet activation and morphology induced by the surfaces and treatment with thrombin or indomethacin, PMN adhesion (Table 3; total adhesion, percentage rolling cells, and rolling velocity) remained unchanged. Additionally, when the flow-oriented platelet aggregates on collagen were turned 90-degrees perpendicular to the PMN flow direction, PMN adhesion did not change (results not shown).

**Discussion**

Exposure of subendothelial tissue to flowing blood results in rapid formation of mixed thrombi consisting of blood platelets and coagulation-induced fibrin.22 Specific proteins expressed in the vessel wall induce the initial platelet adhesion and are main determinants of the subsequent platelet activation with formation of aggregates. Extensive research with components purified from the vessel wall has defined the role of von Willebrand factor, collagen, Fn, fibrinogen, laminin, and other components in this respect.14,23

Recent reports have shown that PMNs minimally adhere to injured vessel wall under flow conditions. The presence of platelets at these surfaces appears to be essential before PMN adhesion can take place. Platelet-associated (P-) selectin is critical for an initial slowing down or rolling interaction. Static leukocyte adhesion follows the interaction of β2-integrins on leukocytes12,13 with platelet-associated ligands as fibrinogen bound to platelet GPIIbIIIa and ICAM-2.8,9 Also fibrin generated by the coagulation cascade and various matrix proteins are ligands for integrins.10,11

So far, only surfaces with nearly confluent coverage (≥80%) of predominantly spread platelets were used to study the role of platelet-associated ligands in PMN adhesion. The influence

<table>
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<th>TABLE 2. PMN Adhesion, Percentage of Rolling Cells, and Rolling Velocity at Platelets Associated With Various Matrix Proteins</th>
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<tr>
<td><strong>ECM</strong></td>
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<tr>
<td>Platelet coverage, %</td>
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<td>PMN adhesion, cells/mm²</td>
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<td>PMN rolling cells, %</td>
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<td>Platelet coverage, %</td>
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<td>PMN adhesion, cells/mm²</td>
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of different platelet coverage and platelet activation on PMN adhesion, however, was not investigated. We therefore studied PMN adhesion on varying platelet coverage and on platelet aggregates. Moreover, we wanted to see whether inhibition of platelet aggregate formation and activation might be a tool to influence the subsequent inflammatory response. In a well-defined perfusion setup, we exposed different models of vessel wall damage to flowing blood, resulting in varying amounts of platelet coverage and activation.

Our experiments show a clear correlation between platelet coverage <15% and subsequent PMN localization. Under these conditions, we observe a characteristic hopping of PMNs from platelet to platelet. At platelet coverage >15%, PMNs are able to roll continuously. Although PMN rolling velocities decrease with increasing platelet coverage, the total PMN adhesion barely increases. The most likely explanation for our results is that a minimal presence of platelet-associated P-selectin enables a continuous rolling interaction, which is necessary before firm and integrin-dependent adhesion can occur. In accordance, we found a comparable effect of platelet coverage on monocyte adhesion. More platelets and thus P-selectin presence only lowers rolling velocity. Lawrence et al reported a similar decrease in rolling velocity when more purified P-selectin was coated on coverslips. The fact that total adhesion is scarcely affected by platelet coverage >15% suggests that platelets do not support integrin–ligand interactions substantially better than the ECM in between. The experiments with purified matrix proteins are consistent with the data obtained with ECM: PMN adhesion is only compromised at platelet coverage <15% (on Fn).

The lower PMN rolling velocity on platelets on FbM is not likely to be caused by increased platelet activation and P-selectin expression because even thrombin was not able to cause this. More efficient integrin–ligand interactions on FbM compared with ECM offers a better explanation. In accordance we showed earlier that PMNs also roll more slowly on platelets with fibrin in between than on platelets on ECM. Rolling velocity at fibrinogen also tended to be lower; however, significance was not reached.

Exposure of injured vessel wall in vivo to flowing blood will within minutes lead to platelet coverage >15%. Very few platelets at microlesions between endothelial cells, however, will mimic the conditions of our low-coverage experiments. In accordance, Kirton et al showed that low platelet adhesion supported PMN adhesion.

Our last goal was to determine whether PMN adhesion was influenced by the activation state of the platelets that were adherent. If the expression of P-selectin should change with this,

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<th>PMN Adhesion, Percentage of Rolling Cells, and Rolling Velocity at Platelets on ECM and Collagen-Coated Surfaces: The Influence of Platelet Aggregate Presence on Collagen and Effect of Additional Platelet Activation by Thrombin and Inhibition by Indomethacin</th>
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<tbody>
<tr>
<td><strong>ECM</strong></td>
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<tr>
<td>Platelet coverage, %</td>
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<td>72±2</td>
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<tr>
<td>PMN adhesion, cells/mm²</td>
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<tr>
<td>Rolling cells, %</td>
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<td>Rolling velocity, μm/s</td>
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Indo indicates indomethacin.
Neutrophil Adhesion at Vessel Wall-Associated Platelets

one would expect parallel changes in PMN rolling velocity as were seen with varying amounts of platelet coverage. Thrombin was chosen as the strong coagulation-dependent platelet stimulus; indomethacin was chosen as a clinically important inhibitor of platelet aggregation. Both had no influence on platelet coverage. In this way, coverage-mediated effects on subsequent PMN adhesion were excluded. Additional platelet activation by thrombin or the aggregates at collagen surfaces changed neither PMN adhesion nor rolling velocity. Inhibition of platelet aggregate formation with indomethacin also had no influence. Hence, we conclude that P-selectin expression on adherent platelets is near maximal and not changed by additional platelet activation or inhibition. Interestingly, rheological differences between platelet aggregates and spread platelets did not influence PMN adhesion. This was clear from the experiments in which the elongated aggregates were turned 90 degrees to the PMN flow direction.

In conclusion, approximately 15% of platelet coverage—no matter whether this coverage consists of spread platelets or platelet aggregates—offers an optimal P-selectin-rich substrate for PMN adhesion. An adhering platelet expresses maximal P-selectin, because additional stimulation or inhibition of these platelets does not change PMN adhesion. An increase in the density of platelet-associated adhesion receptors can result in slower PMN rolling, but this is only obtained by increasing the platelet coverage. Our results indicate that minimal platelet presence, for example at microlesions of the vessel wall, strongly supports leukocyte adhesion. Inhibition of virtually all platelet–vessel wall interactions will be necessary to influence this platelet-induced inflammatory response.

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References

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