Platelet-Monocyte Complexes Support Monocyte Adhesion to Endothelium by Enhancing Secondary Tethering and Cluster Formation


Objective—Adhesion of monocytes to endothelium can be supported by monocyte-monocyte interactions resulting in the formation of cell aggregates at the vessel wall (clusters). Since platelets that are bound to the injured vessel wall support monocyte adhesion and platelet activation in the circulation leads to formation of platelet-monocyte complexes (PMCs), we examined whether adhesion of PMCs to the vessel wall enhances monocyte clustering.

Methods and Results—The effect of PMC formation in monocyte adhesion and clustering on human umbilical vein endothelial cells (HUVECs) was studied in vitro with a perfusion system. In the presence of 10% to 20% PMCs, monocyte adhesion and cluster formation to stimulated HUVECs increased 2-fold above levels obtained with pure monocytes. While the observed effects increased with higher PMC levels, blocking monoclonal antibodies directed against platelet-associated P-selectin or monocyte P-selectin glycoprotein ligand-1 (PSGL-1) reversed adhesion and clustering to control values. In the presence of PMCs, blocking L-selectin decreased adhesion by 25%. When PMCs were present, clustering was only supported by L-selectin at higher shear. These data indicate that monocyte adhesion to the vessel wall is enhanced by PMC-mediated monocyte secondary tethering. These interactions are mainly mediated by P-selectin and PSGL-1.

Conclusion—PMCs in the circulation might be proatherogenic, and prevention of their formation is a possible therapeutic goal. (Arterioscler Thromb Vasc Biol. 2004;24:193-199.)

Key Words: platelet-monocyte complexes (PMC) P-selectin PSGL-1 endothelium atherosclerosis

The recruitment of peripheral monocytes to the site of vascular damage is one of the first steps in atherogenesis and inflammation. However, the interactions between the inflammatory and hemostatic responses as indicated by the colocalization of leukocytes, platelet, and fibrin deposits are also considered essential in this process.

The powerful adhesive interactions that are required for monocytes to withstand local flow at the vessel wall can be described as a multistep process mediated by different adhesion molecules. The initial tethering and rolling of monocytes over the vascular endothelium are mediated by reversible binding of selectins to their cognate cell-surface glycoconjugates. Selectins are expressed on activated endothelial cells (E- and P-selectin), activated platelets (P-selectin), and peripheral leukocytes (L-selectin). Selectins also mediate an additional mechanism for leukocyte adhesion, the so-called secondary tethering. This process involves rolling circulating leukocytes over ligands that are present on surface-adherent leukocytes. The formation of strings or clusters of adhered cells is the result and accounts for the majority of cell accumulation at higher shear stresses. Although this mechanism has been studied more extensively for neutrophil adhesion to endothelial cells, fibrinogen, endothelial cell matrix, and vascular glycoproteins, secondary tethering of monocytes to endothelial cells was also described as L-selectin-dependent.

Although platelets adhered to injured vessel walls form strong adhesive substrates for leukocytes, activated platelets in the circulation also bind leukocytes. These platelet-leukocyte complexes are mostly considered markers of platelet-activating conditions and vessel wall disease, such as unstable atherosclerosis, stable coronary disease, and hypercholesterolemia. Platelet activation was recently reported to induce the development of atherosclerotic lesions of carotid arteries in ApoE mice by increasing the number of vessel wall-adhered leukocytes. Furthermore, an earlier report suggested that platelets bound to monocytoid cells mediate increased adhesion to endothelial cells.
So far, the influence of platelet binding to monocytes on monocyte-monocyte interactions or cluster formation has not been described. We hypothesized that platelets on the monocyte surface enhance monocyte adhesion and clustering at the vessel wall by "bridging" interactions with other monocytes. To test this hypothesis, we perfused isolated monocytes or platelet-monocyte complexes (PMCs) in a transparent perfusion chamber. Real-time image analysis was used to show that platelets on circulating monocytes strongly enhance secondary tethering and, subsequently, monocyte adhesion to stimulated endothelial cells. Our findings suggest the existence of a new proatherogenic pathway in which platelet contribution to leukocyte–leukocyte capture may increase the kinetics of leukocyte recruitment as well as exacerbate inflammation.

Materials and Methods

Reagents

Human serum albumin (HSA) was purchased from CLB Immunoreagentia (Amsterdam, The Netherlands). Reombinant tumor necrosis factor (TNF-α) was purchased from Boehringer Mannheim (Germany). Washing buffer contained phosphate-buffered saline supplemented with 0.5% HSA and 13 mmol/L trisodium citrate. Incubation buffer contained 20 mmol/L HEPES, 132 mmol/L NaCl, 6 mmol/L KCl, 1 mmol/L MgSO4, 1.2 mmol/L KH2PO4 supplemented with 5 mmol/L glucose, 1.0 mmol/L CaCl2, and 0.5% (wt/vol) HSA. Tissue culture supplies (media, antibiotics, and trypsin) were purchased from Gibco, Life Technologies Inc. (Paisley, UK).

Monoclonal Antibodies

Monoclonal antibodies (MoAbs) WASP 12.2 (CD62P, anti-P-selectin), DREG 56 (CD62L, anti-L-selectin), and W6/32 (anti-HLA-A, -B, and -C) were isolated from the supernatant of hybridomas obtained from the American Type Culture Collection (Rockville, Md.). MoAb ENA1 (CD62e, anti-e-selectin) was kindly provided by W.A. Buurman (University Hospital, Maastricht, The Netherlands). MoAbs C17 (CD61, glycoprotein [GP]IIIa), 6C9 (CD41, GPIIb), and MB45 (CD41b, anti-GPIb) were purchased from CLB Immunoreagentia (Amsterdam, The Netherlands). The above-mentioned MoAbs are functionally blocking antibodies. MoAbs PL-1 (blocking of P-selectin glycoprotein ligand-1 [PSGL-1] binding) and PL-2 (nonfunctional blocking) were provided by Dr. Kevin L. Moore (University of Oklahoma, Oklahoma). The CD11a conformation-dependent MoAb CBRM 1/5 was a kind gift of Dr. T.A. Springer (Harvard Medical School, Boston, Mass). All other antibodies were directly FITC-labeled: CD18 (CLB-LFA1/1), CD11a (CLB-LFA1/2), CD11b (CLB-T11.2/1), CD62L (Leu-8, Becton, Dickinson & Company, Franklin Lakes, NJ).

Monocyte Isolation

Whole blood, anticoagulated with 0.4% trisodium citrate (pH 7.4) was obtained from healthy volunteers from the Sanquin Blood Bank (Amsterdam, The Netherlands). Monocytes were isolated from human peripheral blood by means of MACS monocyte isolation kit (Miltenyi Biotech GMBH, Bergisch Gladbach, Germany). This procedure resulted in monocyte fractions containing more than 90% monocytes (CD14-positive cells in FacScan, 15 to 20×106 monocytes were isolated from 50 mL of whole blood, viability exceeding 95% (Trypan blue exclusion). To obtain PMC-poor monocyte suspensions, the monocytes were incubated with a mouse IgG MoAb against GPIIa for 20 minutes at 6°C. After 1 washing step, the cells were incubated with goat-anti-mouse-IgG microbeads (ratio platelets/beads = 1:2; Dynabeads, Dynal A.S., Oslo, Norway) for 20 minutes at 6°C. Magnetic extraction of the beads resulted in a 30% to 40% loss of the initial amount of monocytes and in a PMC presence of less than 5% of the total amount of monocytes. After isolation, the cells were resuspended in HEPES buffer. For blocking experiments, the cells were incubated with MoAbs (10 μg/mL) for 10 minutes at 37°C prior to the perfusion experiments. In some instances, washed platelets were added to the monocyte suspension just before perfusion.

Platelet Isolation

Whole blood was centrifuged at 150 g for 10 minutes to obtain platelet-rich plasma, which was diluted in 1:1 of Krebs-Ringer solution (4 mmol/L KCl, 107 mmol/L NaCl, 20 mmol/L NaHCO3, 2 mmol/L NaSO4, 19 mmol/L trisodium citrate, 0.5% [wt/vol] glucose in H2O, pH 6.1). The mixture was centrifuged at 500 g for 10 minutes, and the supernatant was removed. The pelleted platelets were resuspended in 2 mL of Krebs-Ringer solution and centrifuged at 500 g for 10 minutes. This process was repeated two times, the final suspension being made up in Krebs-Ringer solution to the concentration of 300 000 platelets/μL.

Endothelial Cells

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins as described. The cells were cultured in RPMI 1640 containing 20% (v/v) human serum, 200 μg/mL penicillin, and streptomycin (Life Technologies). Cells were grown to confluence in 5 to 7 days. Endothelial cells of the second or third passage were used in perfusion assays. HUVEC monolayers were activated by TNF-α (100 U/mL, 6 hours, 37°C) prior to the perfusion experiments.

Monocyte Perfusion and Evaluation of Adhesion and Cluster Formation

During perfusions, the flow chamber was mounted on a microscope stage (Axiovert 25, Zeiss, Germany), which was equipped with a B/W CCD video camera (Sanyo, Osaka, Japan) and coupled to a VHS video recorder. Video images were evaluated for the number of adherent cells and the cluster index, with dedicated routines made in the image analysis software Optimas 6.1 (Media Cybernetics Systems, Silverspring, Md). The monocytes in contact with the surface appeared as bright white-centered cells after proper adjustment of the microscope during recording. The cluster index was measured as previously described. In short, the number of cells in an approximate 1750 μm2 surrounding area was measured for each adherent cell. The cluster index per cell was set to be the difference between the measured and the expected number of cells inside an arbitrary area around the cell. For each experiment, the mean cluster index of a minimal 500 cells was calculated.

PMC Quantification and Cell Adhesion Molecule Expression

The percentage of monocytes positive for platelet-specific markers was determined in monocyte suspensions by flowcytometry (FACS Vantage, Becton Dickinson and Company) and also directly in whole blood after lysing the red blood cells. Monocytes from whole blood or bead-isolation were incubated with antibodies against surface markers (GP Ib, GPIbIIa, and CD14) and coupled to a VHS video recorder. Video images were evaluated for the number of adherent cells and the cluster index, with dedicated routines made in the image analysis software Optimas 6.1 (Media Cybernetics Systems, Silverspring, Md). The monocytes in contact with the surface appeared as bright white-centered cells after proper adjustment of the microscope during recording. The cluster index was measured as previously described. In short, the number of cells in an approximate 1750 μm2 surrounding area was measured for each adherent cell. The cluster index per cell was set to be the difference between the measured and the expected number of cells inside an arbitrary area around the cell. For each experiment, the mean cluster index of a minimal 500 cells was calculated.

Statistical Analysis

Data are represented as the mean±SEM of at least 3 independent experiments and were compared with a two-tailed Student t test or a
Platelet Binding to Monocytes and Receptor Expression on Monocytes in Whole Blood and After Isolation.

<table>
<thead>
<tr>
<th>Isolation Procedure</th>
<th>Treatment</th>
<th>Platelets per Monocyte</th>
<th>PMC, %</th>
<th>CD18*</th>
<th>CD11a*</th>
<th>CD11b*</th>
<th>Sle**</th>
<th>CD62L*</th>
<th>CD162*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysed whole blood</td>
<td>Control antibody</td>
<td>ND</td>
<td>10–20</td>
<td>115±14</td>
<td>74±11</td>
<td>38±25</td>
<td>366±33</td>
<td>146±20</td>
<td>111±11</td>
</tr>
<tr>
<td>Monocytes isolated with beads</td>
<td>Control antibody</td>
<td>1±0.3</td>
<td>20–40</td>
<td>126±21</td>
<td>73±23</td>
<td>53±16</td>
<td>325±42</td>
<td>96±38</td>
<td>128±27</td>
</tr>
<tr>
<td>PMC extraction</td>
<td>ND</td>
<td>&lt;5</td>
<td>128±17</td>
<td>84±22</td>
<td>56±21</td>
<td>ND</td>
<td>98±41</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Addition of 3:1 platelets</td>
<td>2.2±0.5</td>
<td>50–70</td>
<td>137±15</td>
<td>87±29</td>
<td>68±19</td>
<td>ND</td>
<td>61±24</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Monocytes isolated by immunobeads were compared to monocytes in RBC-lysed whole blood, in respect to the number of PMCs in suspension and also the number of platelets per monocyte. The mean No. of platelets associated per monocyte was determined by light microscopy (n=3). The percentage of monocytes positive for platelet-specific markers (FITC-labeled GPIb and GPIIbIIIa) was determined by immunofluorescence flowcytometry. Monocytes from RBC-lysed whole blood were also compared to monocytes isolated with immunobeads, considering their expression of various adhesion molecules. The surface marker expression was determined by flowcytometry. ND indicates not determined.

*The data are represented as the mean fluorescence intensity (MFI)±SEM

one-way ANOVA with Bonferroni correction. Probability values <0.05 were considered to be significant.

Results

Quantification and Analysis of PMCs

The presence of monocyte-bound platelets was investigated by flowcytometry and light microscopy. Ten to 20% of the bead-isolated monocytes was associated with platelets, with a mean number of 1±0.3 platelets per monocyte (Table). To confirm that PMC formation also occurs in whole blood, lysed whole blood samples were analyzed with a FACS Vantage (Becton Dickinson). Five to 10% of the monocytes was positive for the platelet-specific markers GPIb and GPIIbIIIa. By adding platelets to the monocyte suspension (platelets:monocyte, 3:1) the mean number of monocyte-bound platelets increased to 2.2±0.5 per monocyte, and the percentage of PMCs increased to 40% to 60%. In contrast, after PMC removal, less than 5% of the bead-isolated monocytes showed binding of platelets (Table).

Surface marker expression of monocytes in whole blood and of isolated monocytes was determined by flowcytometry (Table). When compared with monocytes from lysed blood, beads-isolated monocytes showed some loss of L-selectin, suggesting a slightly stimulated phenotype. This, however, was not significant. Moreover, upregulation of β2 integrins, which indicates that the isolation procedure has triggered cell activation, was not observed. In addition, expression of activated β2 integrins was similar for all monocyte populations (Figure I, available online at http://atvb.ahajournals.org). Also, PSGL-1 and sLeα expression levels were similar after both isolation procedures. Platelets addition did not induce a significant increase in integrin expression on monocytes. However, a decrease in L-selectin expression (from 96±4 to 61±2, P<0.01) on PMCs compared with single monocytes was observed, suggesting that additional platelets activate monocytes to some degree (Figure II; available online at http://atvb.ahajournals.org).

PMC-Associated P-Selectin Mediates Binding of Monocytes to HUVEC

Monocytes in the presence of PMCs showed rolling and firm adhesion to the endothelium. Secondary tethering or cell cluster formation also occurred. By the latter mechanism, flow-oriented trails of cell clusters (Figures 1 and 2) were formed. Both monocytes without platelets on their surface and PMCs were involved in all interactions. However, the influence of PMCs on adhesion and cluster formation became clear from experiments in which PMCs were removed from the monocyte suspension (<5% PMC in suspension). This resulted in a clear decrease in adhesion and cluster formation (Figure 3). PMC-associated P-selectin accounted mainly for this result as the P-selectin–blocking MoAb WASP 12.2 similarly inhibited adhesion by about 60% (711–125 to 304–139 cells/mm2; P<0.001) and clustering by 65% when PMCs were present in the monocyte suspension (Figure 3A). In agreement, under PMC-poor conditions, WASP 12.2 could only reduce adhesion by 30%, while clustering decreased (1.23±0.15 to 0.93±0.05). The presence of P-selectin on the endothelial surface was excluded because preincubation of endothelial cells with WASP 12.2 failed to influence adhesion and clustering (data not shown).

The contribution of E-selectin to monocyte adhesion and/or clustering was also investigated. The blockade of E-selectin on activated endothelial cells inhibited monocyte adhesion by 13%; however, no effect on clustering (data not shown) was observed. Finally, and apart from transient interactions, individual platelets in the monocyte suspensions

![Figure 1](image-url). Platelets promote secondary tethering of monocytes on stimulated endothelial cells. Confocal microscopy detail of a monocyte cluster, formed in the direction of flow, in which platelets (arrows) show bridging interactions with monocytes. Platelets were labeled with FITC GPIb. Bar: 10 μm.
rarely showed firm adhesion to endothelium and therefore did not support monocyte adhesion.

L-Selectin–Dependent Adhesion and Clustering

Although we have shown that in the presence of PMCs P-selectin plays an important role in secondary tethering, L-selectin was first identified to mediate this process.\textsuperscript{11,12}

Therefore, the role of L-selectin was investigated via a L-selectin blocking antibody (DREG 56). In the presence of PMCs, DREG 56 decreased adhesion by 25\% (701±126 to 533±132 cells/mm\(^2\); \(P<0.05\)) (Figure 3B). Clustering, in contrast, was not inhibited by DREG 56 (2.94±0.43 to 3.37±0.99). Under PMC-poor conditions DREG 56 inhibited adhesion by 57\% (338±13 to 148±18 cells/mm\(^2\); \(P<0.001\)) and cluster formation by 42\% (1.2±0.1 to 0.7±0.2; \(P<0.05\)), also at low shear.

As clustering is more important for supporting adhesion at higher shear rates, we repeated these experiments at higher shear stresses (Figure III; available online at http://atvb.ahajournals.org). Although lower adhesion was observed at higher shear, clustering increased up to shear forces of 2.4 dyn/cm\(^2\). At 3.6 dyn/cm\(^2\), the amount of cells that adhered was so low that cluster formation was also hampered. L-selectin did mediate clustering at all higher shear stresses.

Secondary Tethering Is Mainly Mediated by Platelet P-Selectin on PMCs

To better understand the initial events in cluster formation at low shear and the way in which P- and L-selectin are involved, we analyzed images of monocyte perfusions over stimulated endothelial cells and counted the number of PMCs that contributed to clusters and single adhering monocytes.

To estimate this contribution, we counted the total number of platelets bound to single adherent monocytes and the total number of platelets bound to monocytes within the clusters. When monocytes were pretreated with a control antibody we observed a higher amount of platelets bound per monocyte within the clusters compared with the amount of platelets bound to single adhering monocytes (data not shown). This indicates not only preferential contribution of PMCs in the clusters, but also that both PMCs and monocytes without platelets form clusters. The blocking of L-selectin did not affect the p/m ratio within the clusters, but the p/m ratio in single adhering cells increased significantly when compared with the control situation (data not shown).

To better define the role of platelets in secondary tethering, crossover-design-type experiments were performed. Monocytes from a PMC-rich (10\% to 20\% PMCs) or a PMC-poor (<5\% PMCs) suspension were allowed to adhere statically to glass coverslips for 10 minutes at 37\°C. Mounted in the perfusion chamber, the coverslips were then washed with incubation buffer and exposed to PMC-rich or PMC-poor monocyte suspensions at a shear of 0.8 dyn/cm\(^2\). When a PMC-poor monocyte suspension was perfused over PMC-poor or PMC-rich monocyte surfaces, the flowing monocytes did not seem to interact with monocytes/PMCs on the surface because the presence of PMCs on the surface did not affect monocyte adhesion or clustering (Figure 4). However, when PMCs were present in the perfusate an increase in monocyte adhesion and clustering was observed. This effect was increased when PMCs were present on the surface and in the perfusate, suggesting that platelets within the PMCs enhance monocyte adhesion to the endothelium by promoting monocyte secondary tethering.

P-Selectin on Platelets Binds to PSGL-1 Expressed on Monocytes

To investigate the monocyte ligand for P-selectin on platelets, we incubated a PMC-rich monocyte suspension with a
PSGL-1-blocking or nonblocking antibody (PL-1 and PL-2, respectively). Incubation of cells with PL-1 antibody decreased adhesion by 45% (709 ± 71 to 389 ± 40, P < 0.001) and cell clustering by 57% (2.9 ± 0.4 to 1.3 ± 0.1, P < 0.01) (Figure 5). An isotype-matched control antibody (W6/32) or PL-2 showed no effect.

Platelets Augment Monocyte Adhesion and Clustering

To investigate whether we could correlate the amount of PMCs in suspension with the number of firmly adhering monocytes, we compared a monocyte suspension with no PMCs with monocyte suspensions with different amounts of PMCs. Unstimulated and washed platelets (platelet:monocyte ratio used: 1:1, 3:1, 5:1) were added to monocyte suspensions containing 10% to 20% PMCs. Platelet addition to monocytes in all ratios resulted in an increase in PMC formation. An increase in PMC content led to an increase in both monocyte and cluster formations. However, when a 5:1 ratio was used and the percentage of formed PMC increased to 70% to 90%, a decrease in adhesion was observed. This is in agreement with previous results,18 which showed a steep, bell-shaped dose-response curve for leukocyte adhesion to stimulated HUVEC at very low platelet:leukocyte ratios, with a peak at 3 platelets/leukocyte. The blockade of P-selectin decreased adhesion and clustering to basal levels (data not shown).

Possible Platelet-Platelet–Mediated Interactions in Clustering

Subsequently, the potential role of platelet-platelet interactions in bridging PMCs within the clusters was investigated. No influence on monocyte adhesion or clustering by antibody-mediated blockade of the main receptors that mediate platelet-platelet interactions (GPIb and GPIIbIIIa) was observed. Also, addition of the plasma proteins (von Willebrand factor [vWF] and fibrinogen) that bridge the mentioned receptors had no effect (data not shown).

Discussion

The recruitment of monocytes to the vessel wall plays a key role in the pathophysiology of atherosclerosis. Often observed as rolling interactions on the vessel wall, two different types of cell tethering can be discriminated: (1) Primary tethering directly to the endothelial surface and (2) secondary tethering to already adhered cells. Both mechanisms precede firm adhesion of flowing monocytes, but only secondary tethering is associated with cluster formation14 because secondary tethering reduces monocyte velocity, thus facilitating adhesion downstream of adherent cells (Figures 1 and 2). Interactions between L-selectin and sialomucin-like ligands were reported to be responsible for interleukocytic cluster formation.7 In this respect, human monocytes express PSGL-1, a heavily glycosylated sialomucin that can serve as a ligand for L-selectin.8,22 We show here that PMCs, which are markers of platelet-activating conditions, promote monocyte clustering and adhesion to stimulated endothelium. Our experiments show that the formation of monocyte clusters is mediated by platelet-expressed P-selectin and monocyte-expressed PSGL-1. The fact that L-selectin did not mediate monocyte clustering at low shear stress indicates that PSGL-1 is not the main ligand for L-selectin under these conditions. A possible interaction of endothelial P-selectin with the PSGL-1 on monocytes was excluded because preincubation of the
endothelial cells with an antibody to P-selectin did not influence monocyte/PMC adhesion or clustering. Additionally, monocytes themselves do not express P-selectin. Furthermore, blocking the main platelet receptors, GPIb and GPIIbIIIa, which could possibly interact with fibrinogen or vWF in the plasma, also did not affect monocyte adhesion or clustering.

Previous reports have indicated that P-selectin expressed on activated platelets is responsible for an increase in leukocyte adhesion by direct binding to endothelial cells in vitro and in vivo. These interactions were claimed to assist leukocyte adhesion to the vessel wall at shear rates that normally do not allow leukocyte adhesion to activated endothelium. However, intravital microscopy, and also our own observations, characterized interactions of free platelets with endothelial cells as rapidly reversible, leaving doubts about platelet role in leukocyte tethering when bound to their membrane. Theilmeier et al. suggested a PMC role in enhancing monocyte adhesion to the endothelium but used very diluted PMC concentrations that did not result in cluster formation. Our observations show, for the first time, nonrandom homotypic monocyte/PMC clustering in the direction of flow, suggesting a previously unidentified role for platelet in mediating secondary tethering of monocytes.

In addition, PSGL-1 has also been described as being important for leukocyte adhesion to endothelium and as a ligand for E-selectin. However, only a slight decrease (about 13%) in adhesion and no effect on clustering of monocytes/PMC was observed by the blockade of E-selectin. This indicates that PSGL-1, by mediating monocyte-platelet interactions, plays a major role in secondary monocyte tethering (Figure 5). However, we cannot exclude that other adhesion molecules, such as fibrinogen and GPIIb/IIIa, function in the clustering of free-flowing monocytes.

The role of L-selectin on monocyte clustering and adhesion in the presence of PMCs was also investigated. In a PMC-rich monocyte suspension at 0.8 dyn/cm², L-selectin blockade decreased monocyte/PMC adhesion by 25% but did not affect cell clustering. At higher shear rates, however, the role of L-selectin in clustering became more clear. The observation that L-selectin mediates adhesion at all shear rates, irrespective of PMC presence, indicates that monocyte-expressed L-selectin is functionally mediating primary tethering of monocytes to carbohydrate ligands on endothelial cells. L-selectin blockade in the absence of PMCs had a strong inhibitory effect on monocyte adhesion. The latter might be due to the fact that in this situation only E-selectin remains as mediator of initial tethering. In contrast, when PMCs are present, the additional clustering capacity of PMCs as initially adhering monocytes might compensate for the effect of L-selectin blockade on primary tethering and total adhesion. Monocytes with no platelets (and thus no P-selectin expression) on their membrane will, in the case of L-selectin blockade, miss the most prominent selectins for primary interactions with endothelial cells. In contrast, monocytes with platelets bound on their cell membrane are able to use P-selectin or even GPIb and GPIIbIIIa on platelets for primary tethering to endothelial cells. Therefore, the p/n ratio in the single adhering cells increases. The selection and presence of these PMCs on the endothelial surface makes the blockade or L-selectin not critical for monocyte secondary tethering at low shear stresses. These studies provide evidence for an important contribution of circulating activated platelets in the secondary tethering of monocytes at the inflammation site. Cluster formation can, theoretically, start by the adhesion of circulating PMCs to the already-adhered monocytes, but can also follow PMC adhesion to the endothelium, which forms a better platform for secondary tethering of free-flowing monocytes.

Our in vitro experiments show that the clustering mechanism seems capable of clearing the circulation from the free activated platelets. The in vivo fate of platelet/leukocyte complexes in vivo is still unknown. In fact, for a possible accelerating effect of PMCs on atherogenesis, clustered monocytes will have to efficiently penetrate further into the vessel wall. Equally important is the question if platelets bound to monocytes transmigrate with these monocytes into the subendothelial tissue. These questions are currently under investigation.

In conclusion, our results suggest that prevention of platelet activation or platelet interactions with monocytes and, thus, formation of PMC might become an interesting therapeutic approach to modulate atherogenesis.

Acknowledgments
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References


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Expression of activation markers on monocytes derived from different isolation procedures. Monocytes in lysed whole blood and bead-isolated monocytes were incubated with an antibody against L-selectin or an antibody specific for the activated conformation of CD11a (CBRM1/5). Mean fluorescence was determined by flow cytometry.
**Monocyte PMC expression of activation markers after bead isolation procedure.**

Monocytes were isolated from whole blood. PMC-rich and PMC-poor monocyte suspensions were incubated with monoclonal antibodies specific for different adhesion molecules, markers of leukocyte activation. Flow cytometry allowed determination of the mean fluorescence of monocytes with no platelets bound or monocytes within the PMC.
Effect of increasing shear stress on monocyte adhesion and clustering to activated endothelium after blockade of P- or L-selectin. PMC-rich monocyte suspensions were perfused over stimulated endothelium at different shear stresses (0.8, 1.6, 2.4 and 3.6 dyn/cm²). Monocytes were treated with a control (Θ), anti P-selectin (α) or anti L-selectin (Δ) antibody for 5 minutes just before perfusion. Adhesion and clustering behavior of monocytes are expressed in absolute numbers (cells/mm²) and clustering index, respectively. Means ± SEM of 3 experiments are given.