Biology of monocyte interactions with the endothelium: the platelet factor

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Chapter 4

"Platelet binding to monocytes increases the adhesive properties of monocytes by upregulating the expression and functionality of $\beta_1$ and $\beta_2$ integrins"

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Abstract

Human monocytes adhere to activated platelets that display P-selectin, an adhesion molecule that recognizes P-selectin glycoprotein-1 (PSGL-1), which is a specific ligand for P-selectin on leukocytes. We have recently shown that platelet binding to monocytes increases the adhesive capacity of monocytes to activated endothelium. To better understand the effect of platelet binding on the capacity of monocytes to adhere to activated endothelium the P-selectin-PSGL-1 interaction – induced changes in integrin functionality were studied. The binding of platelets to monocytes via P-selectin-PSGL-1 interactions was shown to increase expression and activity of α4β1- and αMβ2- integrin, with a concomitant decrease in L-selectin expression. Furthermore, the binding of platelets to monocytes resulted in increased monocyte adhesion to ICAM-1, VCAM-1 and fibronectin. Platelet binding was also responsible for an increase in monocyte transendothelial migration. Similar effects were observed after engagement of PSGL-1 with specific antibodies or with P-selectin-Ig protein.

Our data suggest that platelets by binding via P-selectin to PSGL-1 on monocytes induce PSGL-1 signaling, leading to upregulation and activation of β1- and β2- integrins and increased adhesion of monocytes to activated endothelium. Hence, monocytes within platelet-monocyte complexes are in a higher state of activation and have an increased atherogenic capacity.
**Introduction**

While monocyte adhesion to the damaged endothelium is essential for atherogenesis, platelet recruitment to atherosclerotic lesions is responsible for acute thrombo-embolic events causing myocardial infarction (1-5). The interactions between platelets and monocytes might therefore modulate both thrombosis and atherogenesis. In this respect, platelet-leukocyte complexes in the circulation are known markers of platelet activation associated with vascular damage caused by atherosclerotic lesions (6,7).

Activated platelets express P-selectin, a member of the selectin family, which upon activation is translocated from the α-granules to the platelet surface (8,9). The main ligand for P-selectin is P-selectin Glycoprotein Ligand-1 (PSGL-1) – a disulfide-linked homodimer with a molecular weight of ~220 kDa on platelets and most leukocytes (10-12). P-selectin and PSGL-1 are considered to be the main players in platelet-monocyte interactions, which not only mediate the binding of leukocytes to activated platelets or thrombi localized at the injured vessel wall but also the formation of platelet-leukocyte complexes (mainly platelet-monocyte complexes) in the circulation (2,13,14).

Monocytes, as other leukocytes, are recruited to cytokine-activated endothelium in a multistep process. Initially, monocytes in the blood stream have to be slowed down by a capturing mechanism and roll over the endothelial layer. Activation of the monocytes during the rolling phase will result in firm adhesion and transmigration (15,17). While the latter process is mediated by interactions between the leukocyte integrins and their endothelial ligands, capture and rolling are mainly mediated by selectins and their respective receptors (17-19). By comparing monocytes and PMC regarding their capacity to adhere to the endothelium, we have previously shown that PMC are more adhesive. This increased adhesion is to a large extent dependent on enhanced monocyte-PMC interactions leading to the formation of flow-oriented monocyte and PMC clusters. P-selectin – PSGL-1 interactions are involved in the formation of these secondary tethers (14).

P-selectin has been shown to increase β2 integrin functionality on neutrophils (20-22) and to enhance the nuclear transcription of NF-κB that is required for the production of cytokines such as MCP-1 and TNF-α (23). The idea of PSGL-1 ligation – mediated signaling has been emphasized by the observation that cross-linking of PSGL-1 on neutrophils induced protein-tyrosine phosphorylation, activated MAP kinases, and stimulated IL-8 secretion. Moreover, PSGL-1 engagement on mouse neutrophils induces LFA-1 - and Mac-1 - dependent adhesion to ICAM-1 (24). Furthermore, P-selectin binding to its counterreceptor PSGL-1 promotes α4β1 – dependent adhesion of monocytes to vascular cell adhesion molecule 1 (VCAM-1) (25).
Altogether these observations suggest a radical change, both quantitative and qualitative, in the leukocyte repertoire of surface-expressed adhesion molecules upon platelet binding. To better characterize the effect of platelet binding on the adhesive capacity of monocytes to adhere to activated endothelium we focused on the P-selectin-PSGL-1 interaction. The present study demonstrates that the binding of platelets to monocytes via P-selectin-PSGL-1 interactions results in stronger adhesion of monocytes to ICAM-1, VCAM-1 and fibronectin. Similar results were obtained after engagement of PSGL-1 with a specific antibody or with P-selectin-Ig. Furthermore, PSGL-1 engagement not only caused changes in integrin activation but it also changed the integrin expression pattern on monocytes.

Our data suggest that platelets by binding, via P-selectin, to PSGL-1 on monocytes, induce PSGL-1 signaling leading to translocation of β1 and β2 integrins to the monocyte surface as well as integrin activation. Hence, monocytes within platelet-monocyte complexes are in a higher activation state and therefore, by adhering more to the endothelium, their atherogenic capacity will be increased.

**Material and Methods**

**Reagents.** Human serum albumin (HSA) was purchased from Sanquin Immunoreagents (Amsterdam, The Netherlands). Bovine serum albumin and PMA were from Sigma-Aldrich (St. Louis, MO, USA). Recombinant TNF-α was from Boehringer Mannheim (Germany) and recombinant MCP-1 was from Strathman Biotech (Hannover, Germany). Alexa Fluor 488 phalloidin and Hoechst were from Molecular Probes (Eugene, OR, USA). Washing buffer contained PBS supplemented with 0.5% human serum albumin and 13 mM trisodium citrate. Incubation buffer contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM MgSO4, 1.2 mM KH2PO4 supplemented with 5 mM glucose, 1.0 mM CaCl2 and 0.5% (w/v) HSA. IMDM medium was from BioWhittaker (Verviers, Belgium) and tissue culture supplies (media, antibiotics and trypsin) were from Gibco, Life Technologies Inc (Paisley, UK).

**Proteins and Monoclonal antibodies.** Human P-selectin-Fc chimera, ICAM-1-IgG and VCAM-1-IgG were purchased from R&D Systems (Minneapolis, MN, USA). Human plasma fibronectin was from Sigma-Aldrich. Monoclonal antibodies (mAbs) WASP 12.2 (CD62P, anti P-selectin), DREG 56 (CD62L, anti L-selectin), IB4 (CD18, anti β2-integrin) and 44A (CD11b) were isolated from the supernatant of hybridomas obtained from the American Type Culture Collection (Rockville, MD, USA). The mAbs as mentioned above are functionally blocking antibodies. Control antibody W6/32 (anti–HLA-A,B,C) was isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection. Specific mAbs against human
PSGL-1, PL-1 (blocking of PSGL-1 binding) and PL-2 (non-blocking) were kindly provided by Dr. Kevin L. Moore (University of Oklahoma, OK, USA). MAb KPL-1 (blocking-PSGL-1 antibody) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MAbs against human CD49d (HP2/1, αβ integrin), VCAM-1 (1G11, CD106) and ICAM-1 (84H10, CD54) were purchased from Immunotech (Marseille, France). The CD11b conformational-dependent mAb CBRM1/5 was a kind gift of Dr. Kevin L. Moore (University of Oklahoma, OK, USA). The β1-integrin conformation-dependent mAb HUTS21 was kindly provided by Dr. F. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain). The following directly labeled mAbs were purchased from Sanquin Immunoreagents (Amsterdam, The Netherlands): IgG1/FITC, IgG2a/FITC, CD14/FITC (clone CLB-mon/1, 8G3), CD62P/PE (clone CLB-thromb/6), CD42b/PE (clone CLB-MB45), CD29/FITC (clone 2A4), CD18/FITC (clone CLB-LFA-1/1), CD11b/FITC (clone CLB-mon-gran/1,B2) and mouse IgG1 (FITC- or PE-labeled). FITC-labeled 44H6 mAb (anti human CD49d) was from Chemicon International (Temecula, CA) and FITC-labeled CD62L (anti L-selectin) was from Becton Dickinson (San Jose, CA). Human and mouse IgG were purchased from Sigma (St. Louis, MO).

**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 negatively selected from human peripheral blood by means of a MACS monocyte isolation kit according to the manufacturer’s instructions (Miltenyi Biotec GMBH, Bergisch Gladbach, Germany). This procedure resulted in monocyte fractions containing more than 90% monocytes (CD14-positive cells in FacScan), the viability exceeding 95% (as determined by Trypan blue exclusion). To obtain PMC-poor monocyte suspensions, the monocytes were incubated with a mouse IgG mAb against GPIIIa for 20 min at 4°C. After one washing step, the cells were incubated with goat-anti-mouse-IgG microbeads (Dynabeads, Dynal A.S., Oslo, Norway), at a ratio of two beads per platelet, for 20 min at 4°C. After magnetic extraction of the beads, the number of PMC was less than 5% of the total number of monocytes. After isolation, the cells were resuspended in incubation buffer. For blocking experiments, monocyte suspensions with or without PMC were incubated with mAbs for 10 min at 37°C prior to the perfusion experiments. In some instances, washed platelets were added to the monocyte suspension just before perfusion (addition of 1 or 3 platelets per monocyte resulted in the formation of 10-20 or 20-40% of PMC, respectively).

**Platelet isolation.** Whole blood was centrifuged at 150 ×g for 10 min to obtain platelet-rich plasma (PRP), which was diluted 1:1 in Krebs-Ringer solution (4 mM KCl, 107 mM NaCl, 20 mM NaHCO3, 2 mM Na2SO4, 19 mM tri-sodium citrate, 0.5%
(w/v) glucose in H2O, pH 6.1). The mixture was centrifuged at 500 xg for 10 min and the supernatant was removed. The platelets in the pellet were resuspended in 2 ml of Krebs-Ringer solution and centrifuged at 500 xg for 10 min. This process was repeated two times, the final suspension being made up in Krebs-Ringer solution to a concentration of 300,000 platelets/μl.

**Cell culture.** Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord veins as described (26,27). Cells were cultured in RPMI 1640 containing 20% (v/v) human serum, 200 μg/ml penicillin and streptomycin (Gibco) and were grown to confluence in 5-7 days. Endothelial cells from the third passage were used in the experiments. TNF-α (100 U/ml) was added directly to the medium 6 hours prior to the experiments. U937 cells (a monocytic cell line derived from human histiocytic lymphoma) were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 (Gibco) containing 10% (v/v) heat-inactivated fetal calf serum (Gibco), 2 mM L-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin (complete medium).

**Adhesion assay.** Adhesion assays were performed as previously described (28,29) with some modifications. Briefly, 96-well microtiter plates (Costar No. 3596, Cambridge, MA) were coated by incubation with fibronectin (10 μg/ml), VCAM-1 or ICAM-1/Fc chimera (1 μg/ml) for 1h at 37°C or 4% HSA as control for 1h at 37°C. After incubation, the wells were washed with PBS and then blocked with 4% HSA at 37°C for 30 minutes. Control wells were filled with 4% HSA in PBS. Monocytes were labeled with calcein AM (Molecular Probes, Eugene, OR, USA) at a final concentration of 5 μg/1 x10^7 cells. In some instances, monocytes were incubated with platelets or P-selectin after calcein labeling. For PMA stimulation, cells were added to wells containing 10 ng/ml of PMA. For blocking experiments, cells were added to wells containing the function-blocking monoclonal antibodies. Plates were then incubated at 37°C and cells were allowed to settle for 30 minutes. After incubation, non-adherent cells were removed by washing twice with PBS and adherent cells were lysed in 0.5% Triton X-100 for 10 min at room temperature. Adhesion was quantified with a microplate fluorescence plate reader (Tecan GENios plus, Tecan Group Ltd, Männedorf, Switzerland). Fluorescence was measured at excitation wavelength 485 nm and emission wavelength 525 nm. The adhesion ratio (%) was calculated as follows: (fluorescence from experimental sample – fluorescence from negative control sample) / total fluorescence added to well * 100%.
Monocyte perfusion and evaluation of cell adhesion. Monocytes in suspension (2 x 10^6 cells/ml in incubation buffer) were aspirated from a reservoir through plastic tubing and perfused through a chamber with a Harvard syringe pump (Harvard Apparatus, South Natic, MA, USA). The shear stress through the chamber was precisely controlled and was kept at 0.8 dyn/cm². During perfusions the flow chamber (27,30) was mounted on a microscope stage (Axiovert 25, Zeiss, Germany), 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 monocytes and the rolling velocity per cell with dedicated routines made in the image analysis software Optimas 6.1 (Media Cybernetics Systems, Silverspring, MD, USA). The monocytes that were in contact with the surface appeared as bright white-centered cells after proper adjustment of the microscope during recording. The number of surface-adherent monocytes was measured after 5 minutes of perfusion at a minimum of 25 randomized high-power fields. To automatically determine the velocity of rolling cells, custom-made software was developed in Optimas 6.1. A sequence of 50 frames representing an adjustable time interval (Δt, with a minimal interval of 80 milliseconds) was digitally captured. The position of every cell was detected in each frame, and for all subsequent frames the distance traveled by each cell and the number of images in which a cell appears in focus was measured. The cut-off value to distinguish between rolling and static adherent cells was set at 1 μm/sec. With this method, static adherent, rolling and free flowing cells (which were not in focus) could be clearly distinguished.

Flowcytometry and confocal microscopy to determine cell adhesion molecules expression upon PSGL-1 engagement on monocytes. Expression of adhesion molecules on the monocyte surface was investigated by flowcytometry (FACS Vantage, Becton Dickinson, San Jose, CA) with cells that were incubated, or not, with platelets (see Material and Methods) or with a P-selectin-Ig. By distinguishing between monocytes with no platelets bound to their surface (CD14-positive/CD42b-negative events) and platelet-bound monocytes (CD14/CD42b-positive events), two different populations of monocytes were characterized, regarding the expression of different adhesion molecules. The expression of CD62L, CD18, CD11a, CD11b, CD29 and CD49d was determined by incubating monocytes with specific, directly labeled antibodies. Isotype-matched control antibodies IgG₁ and IgG₂a were taken along. Integrin activation was investigated by incubating monocytes with the antibodies CBRM1/5 (activation-dependent epitope on α_M subunit) or HUTS 21 (activation-dependent epitope on β₁ integrins).

To further investigate the integrin expression and activation state induced by PSGL-1 engagement by P-selectin, the distribution of α_Mβ₂ (CD11b) and α₄β₁ (CD49d) was characterized by confocal microscopy. Monocytes, untreated or treated with P-selectin-Ig or PMA (positive control) were fixed with 3.7% formaldehyde in
PBS containing 1 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$ for 10 minutes at room temperature. After blocking with PBS containing 0.5% (w/v) bovine serum albumin, 1 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$, CD11a and CD49d were detected with FITC-labeled antibodies. Images were recorded with a Zeiss LSM 510 confocal laser scanning microscope.

**Transmigration assay.** Monocyte transmigration was studied under flow and static conditions. Monocyte suspensions (<5%, 10-20% and 20-40% PMC) were perfused over 6-hour – TNF-α – activated EC. After 5 minutes of perfusion, non-adherent cells were washed away and incubation medium was further perfused for 10 minutes. The adherent cells that migrated through the endothelial cell layer were manually counted every two minutes.

Transmigration assays under static conditions were performed in 6.5-mm, 5 μm-pore Transwell plates (Corning Costar, Cambridge, MA), coated with fibronectin. Freshly isolated monocytes or PMC (1 ×10$^5$) were added to the upper compartment in 0.1 ml of assay medium (IMDM medium with 0.25% (w/v) BSA) and 0.6 ml of assay medium with 10 ng/ml recombinant human MCP-1 were added to the lower compartment. The Transwell plates were then incubated at 37°C, 5% CO$_2$, for different time periods (30, 60, 90 and 120 minutes). Cells that migrated to the lower compartment were collected in a tube to which a fixed number of control U937 cells, labeled with calcein, were added. Flowcytometry analysis was used to determine the ratio between labeled and unlabeled cells. By comparing this ratio with that of the input control, the number of migrated cells was quantified. After the assay cells from the upper side of the filter were removed with a cotton swab. The filters were then fixed and stained with Hoechst. The migrated cells on the bottom side of the filters were counted with a microscope equipped with a UV filter in different fields of the cell filter.

**Statistical analysis.** Data are represented as the mean ± S.E.M. of at least 3 independent experiments and were compared with a two-tailed Student’s t-test or a one-way ANOVA with Bonferroni correction. P values < 0.05 were considered to be significant.
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Results

**PSGL-1 ligation by P-selectin increases monocyte adhesion to immobilized fibronectin, VCAM-1 and ICAM-1.** To test the effect of P-selectin in monocyte adhesiveness, we analyzed changes in monocyte adhesion to fibronectin. Freshly isolated monocytes were treated with various concentrations of P-selectin-Ig and added to fibronectin-coated wells of tissue culture plates. After incubation at 37° C for 30 minutes, unbound cells were washed with PBS, and bound monocytes were quantified. P-selectin-Ig enhanced monocyte adhesion to fibronectin in a concentration-dependent manner. A maximal effect was obtained at 10µg/ml (data not shown).

We further characterized the adhesive capacity of monocytes after PSGL-1 engagement, by platelets or P-selectin-Ig, to various immobilized proteins (fibronectin, VCAM-1, and ICAM-1). Monocytes were incubated with different concentrations of platelets (allowing the formation of PMC, see Material and Methods) or with P-selectin-Ig (10µg/ml). As shown in figure 1A, platelets enhance monocyte adhesion to the different protein surfaces in a concentration-dependent manner. The strongest effect was obtained when 3 platelets per monocyte were added (20-40% PMC). Similarly, binding of P-selectin also enhanced monocyte adhesion to the different surfaces.

To verify the specificity of this effect, i.e., the role of platelets and integrins, the physical interaction of platelets with monocytes was inhibited by antibodies to cell-surface receptors. A blocking antibody against P-selectin blocked the increment in monocyte adhesion to all immobilized proteins (Figure 1B). When monocytes were incubated with HP2/1 (a mAb to integrin α4β1 that blocks leukocyte adhesion) the increment in monocyte adhesion to fibronectin or VCAM-1 was completely abrogated. Similarly, when monocytes were preincubated with IB4 (a mAb to the integrin β2 subunit that blocks leukocyte adhesion) or 44a (a blocking mAb to integrin αM) the monocyte adhesion to ICAM-1 was very low (Figure 1B). A mouse IgG1 antibody had no detectable effect. These data indicate that P-selectin specifically increases monocyte adhesion and suggests that the increased adhesion is mediated by both β1- and β2-integrins.

**Adhesion of monocytes to ECM and endothelial substrates under flow conditions.** We next examined the effects of PSGL-1 engagement on monocyte adhesiveness to fibronectin, VCAM-1, and ICAM-1 under flow conditions. Similar to the results obtained under static conditions, also under flow conditions we observed a significant increase in monocyte adhesion to the different protein surfaces (Figure 2, upper panel).
Figure 1. P-selectin/platelet binding induces monocyte adhesion to fibronectin, VCAM-1 and ICAM-1. Freshly isolated monocytes labeled with calcein, untreated or incubated with platelets (10-20% or 20-40% PMC, see Material and Methods) or P-selectin-Ig (10µg/ml), were added to 96-well tissue culture plates coated without (control) or with fibronectin, VCAM-1-Ig or ICAM-1-Ig (A). For antibody inhibition experiments (B) P-selectin Ig was treated with WASP12.2 (a P-selectin - blocking antibody) prior to incubation with monocytes. Incubation with W6/32 (anti HLA-A, -B and -C, control antibody), HP2/1 (a CD49d – blocking antibody) and IB4 (a CD18 – blocking antibody) or 44A (a CD11b – blocking antibody) was performed after binding of P-selectin Ig to the monocytes and before adding them to the wells on the tissue culture plate. As a control, monocytes were also incubated with mouse IgG (mlgG1), prior to addition to the coated wells. After incubation at 37°C the plates were washed and the bound cells were lysed with 0.5% w/v Triton X-100 for 10 minutes at room temperature. Plates were then read on a microplate fluorescence plate reader at excitation wavelength 485 nm and emission wavelength 525 nm. All results are expressed as the mean ± S.E.M. values of adherent cells / mm² of three independent experiments (**p<0.01, *p<0.05).
To determine whether integrin activation, modulated by PSGL-1 - P-selectin binding, changes the monocyte capacity to adhere to a model of inflamed endothelium we perfused freshly isolated monocytes, incubated or not with platelets or with P-selectin-Ig, over TNF-α - activated HUVEC (Figure 2, lower panel). When the PMC content in the monocyte suspension was less than <5%, blocking antibodies to VLA-4 or Mac-1 (on monocytes) and VCAM-1 or ICAM-1 (on HUVEC) reduced monocyte adhesion by 30%. As expected and as shown before, a P-selectin blocking antibody (WASP12.2) did not have an effect on monocyte adhesion under these conditions. With a PMC content of 20-40%, the same integrin - blocking antibodies also inhibited monocyte adhesion to HUVEC. However, a stronger inhibitory effect (50%) was obtained by blocking P-selectin on platelets.

Figure 2. P-selectin/platelet binding induces monocyte adhesion to fibronectin, VCAM-1 and ICAM-1 under flow conditions. Freshly isolated monocytes, untreated or incubated with platelets (10-20% or 20-40% PMC, see Material and Methods) or P-selectin-Ig (10μg/ml), were perfused over glass coverslips coated with albumin, fibronectin, VCAM-1-Ig or ICAM-1-Ig (A) or with TNF-α – activated HUVEC (B). For antibody inhibition experiments, platelets or P-selectin Ig were treated with WASP12.2 (P-selectin – blocking antibody) prior to incubation with monocytes. Incubation of monocytes with W6/32 (anti HLA-A, -B and -C, control antibody), HP2/1 (CD49d – blocking antibody) or 44A (CD11b – blocking antibody) and of HUVEC with 1G11 (VCAM-1 – blocking antibody) or 84H10 (ICAM-1 – blocking antibody) was performed for 10 min, at 37°, just before starting the perfusion. Results are expressed as the mean ± S.E.M. values of adherent cells / mm² of three independent experiments (**p<0.01, *p<0.05).
Flow cytometric analysis of $\beta_1$ and $\beta_2$ integrin expression on P-selectin- or platelet-bound monocytes. We analyzed the expression of $\alpha_4\beta_1$ and $\alpha_m\beta_2$ integrins on monocytes and determined whether P-selectin binding enhanced the level of integrins expressed on the monocyte surface. PSGL-1 ligation on monocytes was induced by incubation of monocytes with P-selectin-Ig or with different amounts of platelets (see Material and Methods). Platelet- or P-selectin-bound monocytes showed increased expression of both $\alpha_4\beta_1$ and $\alpha_m\beta_2$ integrins (CD49d and CD11b, respectively, Table I, * $p<0.05$) while a decrease in L-selectin expression was observed, suggesting monocyte activation upon P-selectin binding. When P-selectin or platelet binding to monocytes was blocked by incubation of the cells with the anti-P-selectin mAb WASP12.2, no increase in integrin expression was observed, as previously shown (14). Although the increase in integrin expression on platelet-bound monocytes was stronger, an increase in $\beta_2$ integrin expression was also observed on the naked monocytes within the suspensions to which platelets were added.

Table 1. Expression of integrins on monocytes is increased upon platelet/P-selectin binding.

<table>
<thead>
<tr>
<th>adhesion molecule</th>
<th>monocytes</th>
<th>P-selectin Ig (10(\mu)g/ml)</th>
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<tbody>
<tr>
<td></td>
<td>&lt;5% PMC</td>
<td>10-20% PMC</td>
</tr>
<tr>
<td>CD62L</td>
<td>150±14</td>
<td>114±10</td>
</tr>
<tr>
<td>CD18</td>
<td>323±60</td>
<td>404±10</td>
</tr>
<tr>
<td>CD11b</td>
<td>661±24</td>
<td>825±44</td>
</tr>
<tr>
<td>CD29</td>
<td>141±34</td>
<td>128±14</td>
</tr>
<tr>
<td>CD49d</td>
<td>118±14</td>
<td>118±21</td>
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Freshly isolated monocytes (<5% PMC), were incubated with platelets for 30 minutes at 37°C, to allow the formation of platelet-monocyte complexes (PMC, see Material and Methods) or with P-selectin-Ig, (10\(\mu\)g/ml). Incubation of monocytes with a CD42b/PE antibody was used to distinguish two populations of monocytes: monocytes with no platelets bound to their surface and platelet-monocyte complexes (PMC, CD42b positive events). Expression of adhesion molecules was analysed and described in Material and Methods. Specific isotype-matched control (IgG1 and IgG2a) antibodies were taken along. All results are expressed as the mean ± S.E.M. values of mean fluorescence intensity (MFI) of five independent experiments (*$p<0.05$).

Integrin activation was assessed by the use of specific antibodies such as CBRM1/5 and HUTS 21. CBRM1/5 antibody reacts with an activation-dependent epitope on the $\alpha_m$ subunit (Mac-1) while HUTS 21 antibody reacts with an activation-dependent epitope on $\beta_1$ integrins. Incubation of monocytes with platelets or with P-selectin-Ig resulted in 3-5 fold increase in $\beta_1$ and $\beta_2$ integrin activation (Figure 3). In PMC-rich suspensions the naked monocytes did not show an increase in integrin activity.
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Besides the effect of P-selectin binding to PSGL-1, also the influence of specific PSGL-1 antibodies (KPL-1, PL-1 and PL-2) on integrin upregulation on monocytes was investigated. Addition of PL-2 (a non-blocking PSGL-1 antibody), KPL-1 or PL-1 (two blocking mAbs to PSGL-1) to monocytes (no platelets present) also resulted in increased integrin expression and activation (Figure 4). These results indicate that mAb interaction with PSGL-1 on monocytes is sufficient to induce functional up-regulation of β₁ and β₂ integrins.

![Figure 3. Platelet/P-selectin binding to monocytes induces integrin activation.](image)

**Confocal microscopy analysis of integrin expression on P-selectin-bound monocytes.** To further characterize the integrin expression and activation induced by P-selectin binding to the monocytes, the distribution of α₄β₁ and α₅β₂
integrins on the monocyte surface was investigated by confocal microscopy (Figure 5). Non-treated cells (control) stained for CD11b or CD49d showed only a weak and punctuacted staining for both antibodies. As a positive control, PMA stimulation strongly induced a bright staining pattern. Stimulation by P-selectin-Ig chimera resulted in an intermediate staining pattern with the patches being larger and more abundant than in the control conditions. These data indicate induction of variable degrees of avidity of both αMβ2 and α4β1 integrins by P-selectin and PMA.

![Figure 4. PSGL-1 antibodies enhance integrin expression and activation on monocytes.](image)

Washed monocytes were incubated with PL-1 or KPL-1 (blocking mAbs to PSGL-1) or PL-2 (a non-blocking mAb to PSGL-1) antibody. Expression of CD11a, CD11b and CD49d was determined by flowcytometry. Isotype-matched control antibodies (IgG1 and IgG2a) were taken as controls. Integrin activation was analysed by flowcytometry after incubation of cells with antibodies specific for activation-dependent epitopes of CD11b (CBRM1/5) and β1-integrins (HUTS21). Data are expressed as the mean ± S.E.M. values of mean fluorescence intensity (MFI) of three different experiments (\(**p<0.01, *p<0.05\)).

**Effect of platelet binding on monocyte transmigration.** We further investigated a possible correlation between the observed increase in monocyte adhesion to HUVEC upon PSGL-1 ligation and an increase in monocyte transmigration. An increase in monocyte transmigration was observed when 20-40% PMC were present. Under static conditions the percentage of migration towards MCP-1 was 25% higher when PMC were present (Figure 6). PMC presence also influenced monocyte transmigration under flow conditions because 50% of the cells migrated within the first 6 minutes of perfusion while in the absence of PMC this process took 10 minutes (data not shown).
Figure 5. P-selectin induces $\beta_1$- and $\beta_2$-integrin expression and clustering. Monocytes were treated without (control) or with P-selectin-Ig and PMA. Cells were then fixed with 3.7% formaldehyde in PBS containing 1 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$ for 10 minutes at room temperature. After blocking with PBS containing 0.5% w/v bovine serum albumin, 1 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$, cells were incubated with a IgG1, CD11b or CD49d directly-labeled mAbs. Images were taken with a confocal laser scanning microscope. The presented data are representative images of three independent experiments (bar=10 $\mu$m).

Figure 6. Platelet binding to monocytes induces monocyte transendothelial migration. Transmigration of freshly isolated monocytes, untreated (monocytes) or incubated with platelets (PMC, see Material and Methods) was studied under static conditions and under flow. For studies under static conditions, monocytes were added to the upper compartment of a Transwell plate coated with fibronectin and 10 ng/ml of recombinant human MCP-1 was added to the lower compartment. After incubation of the Transwell plates at 37°C for different time periods (30, 60, 90 and 120 minutes), the percentage of migration was quantified. All results are expressed as the mean ± S.E.M. values of percentage of migration or number of migrated cells / mm$^2$ of three independent experiments ($^*p<0.05$)
Discussion

Interactions of PSGL-1 with P-selectin mediate the initial tethering of leukocytes to activated platelets or endothelial cells at sites of infection or tissue injury (13, 31). We recently showed that platelet-monocyte complexes support monocyte adhesion by enhancing secondary tethering (14). In the present study, we extend our previous work by investigating the consequences of platelet binding on the monocyte phenotype regarding expression of β1 and β2 integrins and the adhesive capacity to an inflamed endothelium model. We demonstrated that platelet binding to monocytes induces a high monocyte-activation state, characterized by down-regulation of L-selectin and rapid activation of α4β1- and αMβ2- integrins. Similar effects on monocyte activation were observed after ligation of PSGL-1 by P-selectin-Ig chimera or by specific antibodies to PSGL-1. This P-selectin - triggered integrin activation was completely blocked by a blocking antibody to P-selectin, indicating that physical binding of P-selectin to PSGL-1 on monocytes is essential for this process.

P-selectin - triggered signaling and its stimulatory effects on human leukocytes have been described in several previous reports. P-selectin binding to PSGL-1 has been shown to promote β2-integrin – dependent homotypic neutrophil aggregation and neutrophil-platelet conjugation (22,32). Hidari et al. (24) demonstrated that ligation of PSGL-1 on human neutrophils with mAbs or with P-selectin increased protein tyrosine phosphorylation, activated the ERK MAP kinases and induced secretion of IL-8. Monocytes, upon binding of activated platelets, were shown to secrete monocyte chemotactic protein-1 and IL-8 (33) and express tissue factor (34-36).

Various mechanisms for a role of P-selectin in influencing the activity of β1 and β2 integrins have been suggested. However, most studies have shown that P-selectin cannot directly stimulate integrin activation on human leukocytes (20, 33, 36). Instead, P-selectin was described as an anchoring molecule, allowing monocytes to bind to activated endothelium thus facilitating the binding of EC surface-bound PAF to its receptor on the leukocyte surface. Subsequently, immobilized chemoattractant PAF induced integrin activation (20). Our data indicate that the increase in integrin expression and activation occurs upon PSGL-1 ligation by platelets, P-selectin or by PSGL-1 – specific antibodies. We observed an increase in monocyte adhesion to immobilized fibronectin, VCAM-1 and ICAM-1, indicative of integrin conformational changes. Furthermore, upon platelet binding to monocytes there was an increase in αMβ2 and α4β1 – dependent adhesion of monocytes to activated endothelial cells under flow conditions. This is in agreement with previous studies showing induction of αMβ2 integrin upon platelet binding to human neutrophils.
(20, 21, 37) and an increased affinity of monocytes to VCAM-1 by P-selectin binding under flow conditions (4, 25, 38).

The presence of additional platelet-released synergistic factors such as the cytokines PAF and RANTES (4) seem to be required to induce optimal leukocyte activation. This might explain the observed monocyte adhesion upon P-selectin-Ig binding or PSGL-1 antibodies which was, in some instances, lower than the one obtained by adding freshly isolated platelets to monocytes. A very recent study (20) on neutrophils suggested an intermediate state of integrin activation induced by engagement of PSGL-1 by either P-selectin Ig or antibodies to PSGL-1. This intermediate integrin activation state is compatible with a moderate increase in monocyte adhesion to immobilized proteins or activated endothelial cells. Furthermore, depending on the leukocyte type, PSGL-1 structure and subsequent affinity for its main ligand, P-selectin, seems to differ. When compared to neutrophils, PSGL-1 on eosinophils has been shown to bind 10-fold stronger to P-selectin (39). Recently, platelets were shown to preferentially bind monocytes over neutrophils under flow (40), suggesting differences in structure/affinity of PSGL-1 on the two different cell types. These differences might result in the induction of different PSGL-1-mediated integrin-signaling pathways and explain the effect of platelet binding on both integrin expression and activation observed by us, in contrast to others (20).

In conclusion, our data show an increase in expression and adhesive capacity of $\alpha_4$- and $\alpha_m$-integrins upon PSGL-1 ligation by P-selectin on human monocytes. PSGL-1 ligation by platelet binding results in increased integrin activation and subsequently increased cell adhesion to fibronectin, VCAM-1, ICAM-1 and activated endothelial cells. The concerted action of a variety of stimuli such as chemoattractants, and P-selectin, provided by platelet binding, seems to modulate the activation of monocyte integrins relevant for the monocyte extravasation process and thus for their atherogenic capacity.

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References


Chapter 4. Platelet binding induces activation of integrins on monocytes


