Biology of monocyte interactions with the endothelium: the platelet factor

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Chapter 3

“P-Selectin Glycoprotein Ligand-1 (PSGL-1) is expressed on endothelial cells and mediates monocyte adhesion to activated endothelium”

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Abstract

P-selectin glycoprotein ligand-1 (PSGL-1) is an extensively characterized selectin ligand on leukocytes that mediates interactions with activated endothelial cells (EC). In addition, PSGL-1 plays a crucial role in the formation of platelet-monocyte complexes (PMC). Here we show that PSGL-1 is expressed at the mRNA and protein levels in umbilical vein and microvascular EC. PSGL-1 expression was not affected by treating EC with inflammatory stimuli (TNF-α, IL-1β, thrombin, or histamine). However, the binding of the P-selectin/Fc chimera to endothelial PSGL-1 was significantly increased by TNF-α, indicating that TNF-α modulates the glycosylation of PSGL-1. This was further demonstrated using siRNA strategy to specifically knock-down the genes involved in the glycosylation of PSGL-1. Furthermore, we could demonstrate the contribution of PSGL-1 and its selectin ligands in interactions between monocytes or PMC and activated endothelium in a flow model. Importantly, incubation of activated EC with an antibody to PSGL-1 blocked monocyte adhesion and significantly increased rolling velocity. Similarly, platelet adhesion to activated EC was inhibited when endothelial PSGL-1 or platelet P-selectin were blocked. In conclusion, our results show that EC express functional PSGL-1 which mediates tethering and firm adhesion of monocytes and platelets to inflamed endothelium.
Introduction

P-selectin glycoprotein ligand-1 (PSGL-1) is one of the best characterized selectin ligands. PSGL-1 is a homodimer of two 120-kDa subunits that binds all three selectins, with the highest affinity for P-selectin. Although PSGL-1 was originally identified in human neutrophils and the promyelocytic cell line HL-60, Frenette et al. showed PSGL-1 expression in mouse and human platelets and demonstrated that it mediates platelet-endothelium interactions. Similar to L-selectin, PSGL-1 is constitutively expressed on the surface of most types of leukocytes and plays a role in leukocyte-leukocyte, leukocyte-platelet and leukocyte-endothelium interactions.

Leukocyte and platelet rolling over inflamed endothelium are mediated by the selectin family of adhesion molecules expressed on the endothelium, platelets and leukocytes. P-selectin is stored in granules of EC and platelets and is rapidly translocated to the cell surface after stimulation. Expression of E-selectin on endothelium is induced by inflammatory cytokines such as IL-1β and TNF-α. In contrast, L-selectin is constitutively expressed by leukocytes and is involved in the recruitment of cells into sites of inflammation. In vivo studies showed that leukocyte PSGL-1 mediates rolling of leukocytes over E-selectin on EC while PSGL-1 - L-selectin interactions mediate leukocyte secondary tethering to activated endothelium.

PSGL-1 - dependent interactions appear to bridge the hemostatic and the inflammatory responses. Leukocyte PSGL-1 allows binding to P-selectin on activated platelets, localized at the injured vessel wall. These platelet-leukocyte interactions, mainly between platelets and monocytes, give rise to circulating platelet-monocyte complexes (PMC). PMC are currently regarded not just as markers of vessel wall disease but also as thrombo-atherogenic particles with high adhesive capacity to activated endothelium.

Controversial studies have assessed the presence of PSGL-1 on endothelium. Although Laszik et al. have detected sporadic PSGL-1 expression on endothelium from small venules and capillaries in some pathological studies, Sperandio et al. failed to show PSGL-1 expression on resting or inflamed endothelium and platelets in mice. Therefore, the presence of PSGL-1 on EC has not been further investigated and is currently not considered to be important. We show in this report that PSGL-1 is expressed at the mRNA and protein levels in human vein and foreskin microvascular EC (HUVEC and FMVEC, respectively). Importantly, we also show that endothelial PSGL-1 plays an important role in mediating the rolling and adhesion of monocytes, platelets and PMC over activated endothelium. These findings reveal a new mechanism by which selectins and their ligands participate in the onset of inflammation and/or atherosclerosis.
Material and Methods

**Reagents.** Human serum albumin (HSA) was purchased from Sanquin (Amsterdam, The Netherlands). Recombinant TNFα was from Boehringer Mannheim (Germany) and Texas Red-phalloidin was from Molecular Probes (Eugene, OR). IL-1β was purchased from R&D Systems (Minneapolis, MN). Thrombin and histamine were from Sigma Chemical Co (St. Louis, MO). Recombinant human P-selectin/Fc chimera was from R&D Systems (Minneapolis, MN). Washing buffer contained phosphate-buffered saline (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. Tissue culture supplies (media, antibiotics and trypsin) were from Gibco, Life Technologies Inc (Paisley, UK).

**Monoclonal antibodies.** Monoclonal antibodies (MAbs) WASP 12.2 (CD62P, anti P-selectin), DREG 56 (CD62L, anti L-selectin) and W6/32 (anti HLA-A, B and C; control antibody) were isolated from the supernatant of hybridomas obtained from the American Type Culture Collection (Rockville, MD). 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, Oklahoma). MAb ENA2 (CD62E, blocking of E-selectin binding) was kindly provided by Dr. W. A. Buurman (University Hospital, Maastricht, The Netherlands). MAb against human VCAM-1 (4B2) was purchased from R&D Systems (Minneapolis, MN). The following FITC-labeled MAbs were used: AK-6 anti-CD62P (Sanquin Reagents, Amsterdam, The Netherlands) and CI26CI0B7 anti CD62E (Bender MedSystems, Vienna, Austria). The Alexa-488-labeled goat-anti-mouse-Ig antibody was purchased from Molecular Probes (Eugene, OR) and the FITC-conjugated goat-anti-human-IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Endothelial cells.** HUVEC were isolated from human umbilical cord veins as described

Endothelial cells. HUVEC were isolated from human umbilical cord veins as described. Immortalized HUVEC, EC-RF24 were kindly provided by Prof. H. Pannekoek (Academic Medical Center, Amsterdam, The Netherlands). FMVEC were kindly provided by Prof. V. W. M. van Hinsbergh (VU Medical Center, Amsterdam, The Netherlands). Cells were cultured in RPMI 1640 containing 20% (v/v) human serum, 200 μg/ml penicillin and streptomycin (Life Technologies) and grown to confluence in 5-7 days. Primary endothelial cells from the first, second or third passage were used in the experiments. TNF-α (100U/ml), interleukin-1β (10μg/ml), thrombin (1U/ml) or histamine (1U/ml) was added directly to the medium at different time points prior to the experiments. For blocking experiments, EC were incubated with MAbs for 10 min at 37°C and washed with incubation buffer prior to perfusion.
RNA interference. The mammalian expression vector, pSUPER.retro.puro\textsuperscript{26,27} (a kind gift of Dr. R. Agami, Netherlands Cancer Institute, Amsterdam, The Netherlands) was used for expression of siRNA in HUVEC. The gene-specific insert identifies a 19-nucleotide sequence corresponding to nucleotides 758−777 (gacatctggccacctgcac) of α-4GalT-7 (NM_007255), nucleotides 1037−1056 (atacgccaccgtgcaaac) of GST-1 (NM_003654), nucleotides 1535−1554 (cagcctgtaggagtctaa) of GST-2 (NM_004267), nucleotides 362−381 (gacgacctaccgtagatag) of FX (NM_003313), nucleotides 510−529 (gcacagacctcaaccca) of PSGL-1 (NM_003006), or a sequence with no significant homology to any human gene sequence, therefore used as a non-silencing control. The gene-specific insert was separated by a 9-nucleotide non-complementary spacer (ttcaagaga) from the reverse complement of the same 19-nucleotide sequence, and flanked by restriction sites for the enzymes Bgl II and Hind III, producing a final insert of 60 nucleotides. These sequences were inserted into the pSUPER.retro.puro backbone and transformed into XL Gold supercompetent cells (Invitrogen, USA), according to the manufacturer’s instructions. The different vectors were referred to as pSUPER/α-4GalT-7, pSUPER/GST-1, pSUPER/GST-2, pSUPER/PSGL-1, pSUPER/FX, or pSUPER/Scrambled, respectively. Plasmids were transfected into HUVEC using the Basic Nucleofector Kit for Primary Mammalian Endothelial Cells (Amaxa, Germany) in an Amaxa Nucleofector (Amaxa, Germany), according to manufacturer’s instructions. Immediately after transfection, cells were seeded in glass coverslips coated with crosslinked gelatin (1%) and fibronectin (5 mg/mL). Transfection efficiency was higher than 90% as evaluated by flow cytometry analysis of HUVEC co-transfected pmax/GFP (Amaxa, Köln, Germany) and the different pSUPER constructs (data not shown). To test the efficiency of RNA interference, cells were lysed after 48 h, mRNA isolated (mRNA Capture Kit, Roche, Switzerland) and retrotranscribed into cDNA (Reverse Transcription System, Promega, USA), according to manufacturer’s instructions. Gene expression of α-4GalT-7, GST-1, GST-2, FX, and PSGL-1 was assessed by means of quantitative real-time PCR in an ABI 7900HT platform (Applied Biosystems, USA) using the SYBR Green I chemistry (Applied Biosystems, USA), as previously described\textsuperscript{28}. The primers used were: α-4GalT-7 (Fwd: aggtgaccacctcaggttca, Rev: agtccgtgctgttgctgct), GST-1 (Fwd: ccaagtccagaacagctcactc, Rev: cgccggttgtgatgtcttc), GST-2 (Fwd: gctctgctgctgttcttc, Rev: agagaggtccagcggttaag), FX (Fwd: agccatccagaaggtgtagc, Rev: gacgtggtggtgtggacc), PSGL-1 (Fwd: tgacaccactctctgactgg, Rev: ctccataagctgtgaatcgggt), and GAPDH (Fwd: aggtcatcctgagctgacg, Rev: cgctgtgctcaccacacctg) as endogenous reference gene\textsuperscript{28}.

Isolation of blood cells. 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 by negative selection from human peripheral blood by means of a MACS monocyte isolation kit according to the manufacturer's instructions (Miltenyi Biotech GMBH, Bergisch Gladbach, Germany). This procedure resulted in more than 90% pure monocyte suspensions (measured as CD14-positive cells by flow cytometry). To obtain PMC-poor monocyte suspensions, the monocytes were incubated with a mouse IgG 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 presence of PMC was less than 5% of the total amount 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 and washed in incubation buffer prior to the perfusion experiments.

For platelet isolation, whole blood was centrifuged at 150 g for 10 min to obtain platelet-rich plasma, which was diluted in Krebs-Ringer solution (4 mM/L KCl, 107 mM/L NaCl, 20 mM/L NaHCO3, 2 mM/L Na2SO4, 19 mM/L trisodium citrate, 0.5% (wt/vol) glucose in H2O, pH 5.0). The mixture was centrifuged at 500 g for 10 min and the pelleted cells were resuspended in 2 mL of Krebs-Ringer solution (pH 6.0) and centrifuged at 500 g for 10 min. This procedure was repeated two times, the final suspension being made up in Krebs-Ringer solution (pH 6.0) to a concentration of 10^6 platelets/ml.

**Reverse transcriptase and real-time PCR.** For the reverse transcriptase PCR, total RNA was prepared from freshly isolated monocytes and untreated or IL-1β (4 h) treated HUVEC or EC-RF24 cells with the Absolutely RNA kit (Stratagene). Total RNA (2 μg) was converted to cDNA using 0.5 μg of dT12-18 primer (Invitrogen), Superscript II (Invitrogen) and 20 units of RNAsin (Promega). For the PCR reaction, 5% of the reaction volume served as template for the PSGL-1 primers: FW:5'GGGATCTTCAGGGAAGGAAC3' and Rv:5'CTCCAGTGACCAGGAAGGC3'. The reaction mixture was denatured at 94°C for 2 min and amplified in 35 cycles at 94°C for 15 s, 60°C for 20 s and 72°C for 45 s. PCR products were resolved on a 1.5% agarose gel.

**Western blotting.** Monocytes and HUVEC were lysed in 15% Triton X-100, 0.1% SDS, 0.1% NP-40, 100 mM Tris-HCl pH 7.4, 150 mM NaCl, and 1 mM CaCl2 buffer. Proteins from the cell lysates (1×10^6 monocytes and 2×10^6 HUVEC) were separated on 7% SDS-PAGE, transferred to a PVDF membrane and blotted with PL-1 antibody. The bound antibody was detected by using HRP-conjugated secondary antibody.
Flowcytometry and confocal microscopy. PSGL-1 surface expression on EC was investigated by flowcytometry (FACS Vantage, Becton Dickinson and Company, CA) with cells from different passages, stimulated or not with TNF-α (10 and 30 min, 2, 6, 12 and 24 h), IL-1β (6 h), thrombin (5 and 10 min) or histamine (5 and 10 min). After stimulation, EC were resuspended in washing buffer and incubated with a control antibody (FITC-labeled goat anti-mouse IgG), or an antibody against PSGL-1 (PL-1 or PL-2), P-selectin (AK6) or E-selectin (CI26CI0B7) for 45 min at 4°C. Cells were washed with washing buffer before analysis. For confocal microscopy, EC seeded on fibronectin-coated glass coverslips were immediately stained and analyzed or fixed with 3.7% formaldehyde in PBS containing 1 mM Ca\(^{2+}\) and 1 mM Mg\(^{2+}\) for 10 min at room temperature. After blocking with PBS containing 0.5% bovine serum albumin, 1 mM Ca\(^{2+}\) and 1 mM Mg\(^{2+}\), PSGL-1 was detected with PL-1 antibody followed by an Alexa-488-labeled goat-anti-mouse-Ig antibody. In a similar way, specific antibodies were used to detect VCAM-1 and E-selectin on EC.

The affinity of endothelial PSGL-1 for P-selectin was tested with a P-selectin/Fc chimera. EC, activated or not with TNF-α, were incubated with P-selectin/Fc chimera for 20 min at 37°C. After washing, the cells were prepared for flowcytometry and confocal microscopy, as described above. P-selectin/Fc protein binding to EC was detected with a Alexa Fluor 488-conjugated goat-anti-human-IgG antibody. Fixed cells were counterstained for F-actin with Texas Red-phalloidin. Images were recorded with a Zeiss LSM 510 confocal laser scanning microscope.

Monocyte perfusion and evaluation of adhesion and rolling velocity. 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). The flow rate through the chamber was precisely controlled and the monocytes were perfused over EC at 0.8 dyn/cm^2. During perfusions the flow chamber 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 min 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 \( \mu \text{m/s} \). With this method, static adherent, rolling and free flowing cells (which were not in focus) could be clearly distinguished.

**Platelet perfusion and evaluation of adhesion.** Platelets in suspension (10\(^6\) cells/ml in Krebs-Ringer buffer), incubated with a control antibody (W6/32) or with a P-selectin blocking antibody (WASP 12.2), were stained with green calcein (Molecular Probes, Eugene, OR) and perfused over EC in the same way as for monocytes (see above) with minor modifications. In short, the flow rate through the chamber was maintained at 6 dyn/cm\(^2\) and the flow chamber was mounted on a Zeiss LSM 510 confocal laser scanning microscope. Images of at least 60 different fields were taken. For every image the adhered platelets were manually counted.

**Tissue specimens and immunohistochemistry.** Portions of coronary arteries were obtained from autopsy specimens at the University Hospital of Utrecht (The Netherlands) and were procured according to institutional guidelines. Coronary arteries undergoing atherosclerosis were snap-frozen and seccioned using conventional techniques. Slides were kept for 20 minutes at room temperature before staining. Histochemical staining for PSGL-1 antigen was performed by means of avidin biotin peroxidase methodology. The slides were incubated with PBS containing 10% horse serum to inhibit nonspecific antibody binding, followed by primary antibody diluted in PBS/1% bovine serum albumin for 45 minutes. The working concentration of the PL-1 MoAb was 2.5 \( \mu \text{g/ml} \). An irrelevant mouse monoclonal IgG\(_1\) was used at equivalent concentration (Sanquin Reagents, Amsterdam, The Netherlands). Slides were then incubated with biotin-conjugated goat anti-mouse secondary antibody for 30 minutes, followed by a chromogen/substrate reagent solution (diaminobenzidine/H\(_2\)O\(_2\), Sigma) for 10 minutes. Hematoxylin was used for nuclear counterstaining. Between all incubation steps the slides were washed with PBS. For each antibody, three different specimens were analysed.

**Statistical analysis.** Data are represented as the mean \( \pm \) 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.
Results

**PSGL-1 is expressed in endothelial cells at the mRNA and protein levels.**

Because PSGL-1 is involved in leukocyte-endothelium interactions and PSGL-1 has been suggested to be present in the endothelium of small venules and capillaries of some pathological tissues, we investigated whether PSGL-1 is also expressed on EC. We first evaluated with RT-PCR the presence of PSGL-1 mRNA in untreated and TNF-α or IL-1β - treated primary HUVEC and in EC-RF24 cells. RNA from freshly isolated monocytes was used as a positive control. After reverse transcription and DNA amplification, a predicted 240-bp PCR product was obtained from HUVEC (Figure 1A) and EC-RF24 cells (data not shown) with no detectable differences between stimulated and unstimulated cells. A similar PCR product was also amplified from monocyte RNA. As a positive control for TNF-α or IL-1β stimulation we analyzed ICAM-1 mRNA (data not shown) which showed a dramatic increase in expression in response to these cytokines.

The expression of PSGL-1 protein in EC was also analyzed by Western blot analysis (Figure 1B). Although the level of PSGL-1 protein in EC was much lower than in monocytes, a protein of similar apparent molecular weight was observed in both cell types. Flow cytometric analysis further showed that PSGL-1 is expressed on the surface of EC (Figure 1C). In contrast to E-selectin, there was no increase in surface levels of PSGL-1 following activation of the EC with TNF-α (Figure 1C) or IL-1β (data not shown). Different incubation times with TNF-α (10 or 30 min, 2, 6, 12 or 24 h) also did not affect the level of expression of PSGL-1 (not shown). PSGL-1 expression levels on FMVEC were similar to the ones obtained for HUVEC (Figure 1D). Finally, no changes in endothelial PSGL-1 expression were detected when EC from the first, second and third passages were compared (data not shown).

To analyze whether PSGL-1 surface expression could be upregulated by recruitment from intracellular stores, as is known for P-selectin, EC were stimulated with thrombin or histamine for 5 or 10 min (Figure 1E). In contrast to the increase in P-selectin expression (positive control), no difference in PSGL-1 expression was detected upon treatment with these stimuli. Immunofluorescent analysis of PSGL-1 (Figure 2) on untreated and TNF-α activated HUVEC further confirmed that PSGL-1 is expressed on the endothelial cell surface and that its expression is not altered by cell activation. In contrast, expression of VCAM-1 and E-selectin was detected only after endothelial activation.
Figure 1. PSGL-1 mRNA and protein are present in endothelial cells. (A) Presence of PSGL-1 mRNA in HUVEC and monocytes. For reverse transcriptase PCR, total RNA was prepared from untreated HUVEC, 6 h-IL-1β-treated HUVEC and human monocytes. Gene-specific primers were used to amplify cDNA fragments for PSGL-1. A fragment of the same length was obtained from the different cells (arrow, ~240 bp). The marker and the product of the reaction without reverse transcriptase (-RT) are indicated. (B) PSGL-1 protein expression on HUVEC was determined by Western blot. Lysates from monocyte, purified from whole blood, or from stimulated HUVEC were analysed and showed a protein of similar molecular weight (arrow, ~120 kD). The effect of cytokine stimulation on PSGL-1 expression on HUVEC (C) and FMVEC (D) was analysed by flowcytometry. Cells were left untreated or were incubated with a control mouse IgG1 (control), PL-1 (PSGL-1) or CI26C10B7 (E-selectin) FITC-labeled antibody for 45 min at 4 °C. Data shown are representative of 3 or more experiments. (E) HUVEC were treated with thrombin (1 U/mL) or histamine (1 U/mL) for 5 or 10 min. Cells were incubated with a control mouse IgG1 (control antibody, empty bars), PL-1 (blocking anti-PSGL-1 antibody, filled bars) or PL-2 (non-blocking anti-PSGL-1 antibody, hatched bars) or with WASP12.2 (blocking anti-P-selectin antibody, gray bars) and analyzed by flowcytometry. Data represent the mean ± SD (n=4).

Endothelial PSGL-1 can bind P-selectin and mediate platelet adhesion to endothelium. To determine whether PSGL-1 expressed on EC is able to bind P-selectin, platelets were perfused over EC and adhesion was quantitated. Washed and labeled platelets were incubated with a control (W6/32) or a blocking P-selectin antibody (WASP 12.2) and perfused at high shear over untreated or TNF-α-treated (6h) EC. Platelet adhesion was strongly increased (90 %) following activation of EC (Figure 3). This effect was strongly inhibited when PSGL-1 on activated EC or P-selectin on platelets was blocked with PL-1 or WASP12.2 antibodies, respectively.
Although similar amounts of PSGL-1 are present on the surface of unstimulated and stimulated EC, apparently only stimulated cells are able to support platelet adhesion. In order to test whether there is an increase in PSGL-1 affinity for its receptor upon stimulation, a P-selectin/Fc chimeric protein was allowed to bind to unstimulated and TNF-α-stimulated EC. Analysis by flow cytometry and immunofluorescence (Figures 4A and 4B, respectively) showed that the P-selectin/Fc protein bound significantly more to stimulated than to unstimulated EC. The binding of the P-selectin/Fc chimera to PSGL-1 was inhibited by a blocking antibody to PSGL-1, which underscored the specificity of the interaction between the P-selectin/Fc chimera and PSGL-1. These results indicate that, despite PSGL-1 being constitutively expressed on EC, the affinity for its receptor can be increased by cytokine stimulation of the endothelium.

**Figure 2. Immunolocalization of PSGL-1 on endothelial cells.** Fixed (A) and unfixed HUVEC (B) were treated or not with TNF-α for 6 or 18 h and PSGL-1 expression was detected by confocal microscopy. PSGL-1 was detected with PL-1 antibody (blocking antibody directed against PSGL-1) followed by an Alexa-488-labeled goat-anti-mouse-Ig antibody. In a similar way, specific antibodies were used to detect VCAM-1 and E-selectin on HUVEC. Cells were counterstained for F-actin with Texas Red-phalloidin, which is shown in red. PSGL-1, VCAM-1 and E-selectin are in displayed in green. (bar: 20 μm).
Endothelial PSGL-1 mediates the initial tethering and firm adhesion of monocytes and PMC to endothelial cells. To investigate whether endothelial PSGL-1 is also functional in mediating monocyte and platelet-monocyte complex (PMC) interactions with the endothelium under flow, monocytes were perfused over HUVEC (untreated or stimulated with TNF-α for 6 h). Video recordings were analyzed for the number of adhered monocytes and for rolling velocity (see Material and Methods). Perusions of monocytes or PMC over unstimulated EC resulted in very little monocyte adhesion (data not shown). Therefore, subsequent experiments on the role of PSGL-1 in monocyte or PMC recruitment to endothelium were performed with cytokine-activated EC. In the presence of relatively high levels (10-20%) of PMC, blocking PSGL-1 or P-selectin on monocytes or PMC significantly inhibited monocyte adhesion by 30% (from 1124 ± 132 to 790 ± 18 cells/mm², p < 0.05) and strongly increased monocyte rolling velocity (Figure 5). Simultaneous inhibition of PSGL-1 on EC and on monocytes caused a synergistic reduction of monocyte adhesion (data not shown). To test the role of endothelial PSGL-1 in the adhesion of monocytes in the absence of platelets, PMC were removed from the cell suspension by immunodepletion. As previously reported, low levels of PMC (< 5% PMC in suspension) resulted in reduced monocyte adhesion to the endothelium. By blocking PSGL-1 on EC, monocyte adhesion was further decreased 30% (Figure 5A), while rolling velocity was significantly increased (Figure 5B). As was shown before, blocking of P-selectin on the endothelium did not have an effect in cell adhesion.

![Graph](image-url)

**Figure 3.** PSGL-1 mediates platelet adhesion to TNF-α activated HUVEC. Platelets in suspension were labeled with calcein, washed and perfused over untreated or 6 h-TNF-activated HUVEC for 5 min at 6 dyn/cm². Video images of at least 60 different fields were taken per experiment. For every image the number of platelets adhered was manually determined. Where indicated, platelets were treated prior to perfusion with an antibody to P-selectin (WASP12.2). Similarly, endothelial cells were treated or not with a blocking antibody to PSGL-1 prior to perfusion. Data represent the mean ± SD (n = 4, * p < 0.05).
Figure 4. PSGL-1 affinity to P-selectin/Fc protein. Untreated or TNF-α (6 h) – stimulated cells were incubated with a P-selectin/Fc chimera for 20 min at 37 °C. Where indicated, the cells were incubated with a blocking antibody to PSGL-1 (PL-1). Protein binding to the endothelial cells was detected with a FITC-conjugated goat-anti-human-IgG antibody by flowcytometry (A) control (dotted line), unstimulated EC (regular line) and stimulated EC (thick line)) or by immunofluorescence confocal microscopy (B). Data are shown as the representative of three experiments. (bar: 20 μm).

Figure 5. PSGL-1 functionality on monocyte adhesion to TNF-α activated endothelium. PMC-rich (10-20 % PMC, filled bars) and -poor (< 5 % PMC, empty bars) monocyte suspensions were perfused over TNF-α activated (6h) HUVEC for 5 min at 0.8 dyn/cm². Video images were evaluated for the number of adherent monocytes (A) and cell rolling velocity (B). Prior to perfusion, HUVEC were incubated either with W6/32 control antibody, with PL-1 (blocking antibody to PSGL-1), or WASP12.2 antibody (blocking antibody to P-selectin). Data represent the mean ± SD (n = 3, * p < 0.01).
To investigate whether endothelial PSGL-1 can interact with L-selectin on monocytes, an L-selectin-blocking antibody was used on a monocyte suspension containing < 5% PMC. To rule out a possible contribution of remaining platelets, the monocytes were, where indicated, incubated with an antibody to P-selectin to prevent PMC formation. When the cells were incubated with the DREG 56 antibody to L-selectin, adhesion to the endothelium was inhibited by 35% (from 683 ± 107 to 439 ± 61 cells/mm², *p < 0.05), (Figure 6A). This effect was similar to that obtained by blocking PSGL-1 on EC. Although not statistically significant, when both L-selectin on monocytes and PSGL-1 on EC were blocked, monocyte adhesion was further inhibited. As a control we used a non-blocking antibody against PSGL-1 (PL-2) which did not affect monocyte adhesion to the endothelium (data not shown).

Figure 6. PSGL-1 functionality on monocyte adhesion to TNF-α activated endothelium.
A. PMC-poor (< 5% PMC) monocyte suspensions were pretreated with WASP12.2 anti-P-selectin antibody prior to incubation with other antibodies. The monocytes were perfused over TNF-α-activated (6 h) HUVEC for 5 min at 0.8 dyn/cm². Video images were analyzed for the number of adhered cells. Prior to perfusion, cells were incubated with W6/32 control antibody (monocytes and endothelial cells), anti L-selectin DREG 56 antibody (monocytes) or anti PSGL-1 PL-1 antibody (endothelial cells). Data represent the mean ± SD (*p < 0.05) .

B. PMC-poor (< 5% PMC) monocyte suspensions were perfused over transfected-TNF-α-activated (6 h) HUVEC for 5 min at 0.8 dyn/cm² (see RNA interference in Material and Methods section). Video images were analyzed for the number of adhered cells. Prior to perfusion, cells were incubated with anti PSGL-1 PL-1 antibody (endothelial cells). Data represent the mean ± SD (*p < 0.05).
Previously it has been shown that the expression of L-Selectin ligands in endothelial cells is modulated by sulfation, and that TNF-α upregulates the expression of two sulfotransferases implicated in the sulfation of L-Selectin ligands. To investigate whether the mechanism of increase in monocyte adhesion described here is dependent on the sulfation of PSGL-1 an RNA interference approach was designed. The genes targeted were GST-1 and -2, implicated in the sulfation of N- and O-linked glycans, α-4GalT-7, involved in the initiation of the glycosaminoglycan chains, and FX, which controls the synthesis of GDP-Fucose. Additionally, a knockdown for PSGL-1 and a sequence without homology in the human genome were used as a positive and negative control, respectively. Importantly, the silencing of PSGL-1 results in a decrease in monocyte adhesion (Figure 6B) and an increase in rolling velocity (data not shown) which are comparable to the effect of blocking with PL-1 (Figure 6B). Furthermore, the silencing of GST-1 was able to mimic the effects of silencing PSGL-1, while any of the other treatments were ineffective (Figure 6B).

Together, these data reveal an important role for endothelial PSGL-1 in monocyte and PMC adhesion to the vascular endothelium via direct interaction with P- and L-selectin on platelets and monocytes, respectively.

**PSGL-1 expression in atherosclerotic coronary arteries.** Sections of coronary arteries undergoing acute inflammation such as atherosclerosis were examined for PSGL-1 antigen. Expression of VCAM-1 and ICAM-1 was analysed as a positive control and a strong staining of the vascular endothelium was observed with the ICAM-1 and VCAM-1 MoAbs. Although to a much lower extent, the sections also exhibited luminal staining with the anti PSGL-1 MoAb indicating clear PSGL-1 expression on the vascular endothelium of these arteries. In contrast, staining of the endothelium with an irrelevant mouse IgG1 MoAb was not detected.

**Discussion**

The molecular mechanisms by which leukocyte recruitment to inflamed tissues occurs have been extensively studied over the past years. The initial tethering and rolling of monocytes along the vessel wall is generally accepted to be mediated by selectins and their ligands that are expressed on EC, platelets and leukocytes. PSGL-1, one of the primary selectin ligands, is known to be expressed on leukocytes and platelets. In this study we show that functional PSGL-1 is expressed on EC upon treatment with proinflammatory cytokines.
Figure 7. Immunohistochemical analysis of PSGL-1 antigen in the vascular endothelium of atherosclerotic coronary arteries. Snap-frozen sections of coronary arteries undergoing atherosclerosis were incubated with an irrelevant IgG, antibody or antibodies against ICAM-1, VCAM-1 or PSGL-1. Bound antibody was detected using avidin biotin peroxidase methodology (see Material and Methods section). VCAM-1 and ICAM-1 strongly stained the endothelium of the artery. Although to a less extent, PSGL-1 staining of the endothelium as well as intravascular leukocytes was detected.

A 240-bp product was obtained by reverse transcriptase PCR with PSGL-1-specific primers on RNA from primary EC or from an endothelial cell line. Protein expression was confirmed by western blot analysis of endothelial cell lysates. Flowcytometry analysis and confocal microscopy further confirmed PSGL-1 protein expression on the surface of primary HUVEC and FMVEC. Surprisingly, we found that PSGL-1 surface expression on EC is not increased by stimulation with inflammatory cytokines such as TNF-α or IL-1β. This is in marked contrast to the cytokine-induced upregulation of the endothelial adhesion molecules ICAM-1, VCAM-1 and E-selectin. In addition, activators such as thrombin or histamine, which induce elevated surface expression of P-selectin on EC, had no effect on the expression levels of either PSGL-1 mRNA or protein. Thus, PSGL-1 is constitutively expressed in primary EC and in immortalized endothelial cells, and is not stored in P-selectin-containing vesicles within EC.

Endothelial PSGL-1, at higher shear stress, was able to interact with platelets and recruit them to the endothelium. This effect was inhibited by blocking P-selectin on platelets or PSGL-1 on EC. The increase in affinity of endothelial PSGL-1 to P-selectin was further demonstrated by the strong binding of a P-selectin/Fc protein to stimulated HUVEC, which was abrogated by incubating the cells with a blocking antibody to PSGL-1. Although PSGL-1 is expressed on both untreated and cytokine-
treated EC, platelet adhesion was only observed upon cell activation. Vachino et al. have shown that, although most lymphocytes express PSGL-1, only 10-20% of cells are able to bind P-selectin, which shows that the presence of PSGL-1 on the cell surface is not equivalent to functional relevance. PSGL-1 needs to be tyrosine sulfated and properly decorated with core 2 - based O-glycans expressing sialyl Lewis x to be functional. Differences in the number of core 2 – based O-glycans or in the presence of lactosamine repeats, as well as sulfation, fucosylation or sialylation, affect the recognition of PSGL-1 by the selectins. Here we show that silencing of the sulfotransferase GST-1, and partially GST-2, mimics the effect of silencing PSGL-1 or using the blocking antibodies PL-1 or DREG 56. Furthermore, silencing of α4GalT-7 or FX had no effect on the adhesion of monocytes to activated EC. Altogether, these data indicate that TNF-α-induced expression of functional PSGL-1 is dependent on the expression of GST-1, and partially GST-2, while fucosylation or the expression of glycosaminoglycans might not be of importance. These findings are in line with those of Li et al., demonstrating the critical role of GST-1 and -2 in shear-resistant leukocyte rolling via L-selectin. Finally, a cytokine-induced increase in affinity of endothelial PSGL-1 for its receptor is also suggested by the enhanced binding of the P-selectin/Fc chimera to EC after stimulation. This assay reflects binding to PSGL-1, as this is the only receptor for P-selectin on the EC. The expression of various other adhesion molecules involved in leukocyte adhesion to stimulated endothelium might explain the observation that the inhibition of endothelial PSGL-1 never resulted in complete inhibition of monocyte adhesion.

Platelets are known to form platelet-monocyte complexes (PMC) via P-selectin - PSGL-1 interactions. Monocyte isolation results in a monocyte suspension containing 10-20 % PMC. PMC have been reported to support monocyte adhesion to endothelium by enhancing secondary tethering. Therefore, we performed blocking studies with anti-PSGL-1 antibody to investigate the tethering and adhesive behavior of monocytes/PMC. Our flow system enabled us to show functionality of endothelial PSGL-1 as a ligand for selectins on monocytes and platelets. When 10-20 % PMC were present in the monocyte suspension, we found a significant reduction (30 %) in monocyte adhesive interactions with the endothelium, accompanied by an increase in cell rolling velocity when TNF-α - stimulated EC were pre-incubated with a PSGL-1 - blocking antibody.

Under low shear conditions, platelet interactions with the endothelium are mainly characterized by transient tethering and rolling, whereas firm adhesion rarely occurs. However, it is important to discern whether PSGL-1 on EC interacts mainly with L-selectin on monocytes or might also interact with P-selectin on platelets. To investigate this, we have removed PMCs from the monocyte suspension. By blocking PSGL-1 on EC or L-selectin on monocytes, we were able to increase monocyte rolling velocity and inhibit monocyte adhesion to the endothelium by 30 %, indicating that monocyte L-selectin is a primary receptor for endothelial
PSGL-1. Although not statistically significant, our results show that simultaneous blocking of endothelial PSGL-1 and L-selectin on monocytes results in further inhibition of monocyte adhesion. This might be explained by the fact that L-selectin is known to bind also other ligands on the endothelium, such as CD34 and MAdCAM-1. In addition, by blocking L-selectin on monocytes, cell adhesion might be affected not only because the interaction between L-selectin on monocytes and endothelial PSGL-1 is inhibited but also because primary tethering of monocytes over the endothelium is inhibited.

We show that primary EC constitutively express functional PSGL-1. Our results are, to a small extent, in agreement with the immunohistochemical analysis of Laszik et al. in which, only sporadic PSGL-1 expression on endothelium of small venules and capillaries in some pathological tissues was described. When analyzing EC-surface expression of PSGL-1 by confocal microscopy we detected some antigen loss after cell fixation with paraformaldehyde. Therefore, the immunohistochemical discrepancies observed by us might be due to the fact that we analysed snap-frozen material and thus prevented possible antigen loss by paraformaldehyde treatment.

In conclusion and although expressed at low levels, PSGL-1 on activated EC is able to functionally bind P- and L-selectin on platelets and monocytes, respectively, mediating monocyte initial tethering and platelet recruitment to the endothelium. Our results strongly suggest that PSGL-1 has a crucial role in monocyte/PMC and platelet recruitment to the vascular endothelium and should be considered as an important participant in the onset of inflammation and/or atherosclerosis.
Chapter 3. Endothelial PSGL-1 mediates monocyte adhesion

References


