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POTENTIAL ROLE OF P-SELECTIN GLYCOPROTEIN LIGAND-1 IN HAEMATOLOGICAL DISEASES

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Summary: PSGL-1 is a major counterreceptor of all three types of selectins that is expressed in several leukocyte subsets. Data presented, here prove that this mucin may be implied in haematological disorders. We established on normal peripheral blood and in samples derived from 20 AML patients that PSGL-1 is differently expressed in various leukocyte subsets. Myeloblasts appearing in acute myeloid leukaemia patients express significantly less PSGL-1 (12 000 ± 5300) than mature neutrophils ($p < 0.001$). In monocytic leukaemias, however, the amount of PSGL-1 on monocytic precursors is displayed in a fairly broad range which was not significantly different from that of mature monocytes ($p = 0.084$). Monoblasts/promononocytes possess more PSGL-1 than myeloblasts and the expression pattern is completely non-overlapping. This would imply a differential expression of PSGL-1 during myeloid haemopoietic development and suggests, that the quantitation of surface PSGL-1 may help in differentiating myeloblasts from monoblasts by immunophenotyping in different AML subsets. PSGL-1 has also a certain role in the generation of procoagulant microparticles (MP) as in the PSGL-1 knockout mouse the MP number failed to increase with age and the MP contained significantly less tissue factor than wild type mice. Since PSGL-1 P-selectin interaction is crucial in generating a procoagulant effect we tested the hypothesis that the administration of a P-selectin IgG chimera (Psel-Ig) corrects bleeding tendency in a murine haemophilia model and in human haemophilic blood. The addition of Psel-Ig resulted in significant improvement of the bleeding tendency in mice and in the generation of MP in human hemophilic blood. Thus, the Psel-Ig can become an alternative route to control bleeding tendency in coagulopathies.

Key words: P-selectin glycoprotein ligand 1, acute myeloid leukemia, hemophilia

Introduction

Leukocytes express a vast array of receptors, that play an important role in heterotypic cell contact. These surface molecules enable leukocytes to interact with each other as well as with platelets and endothelial cells. It has been suggested, that there are several similar processes in leukocyte and platelet rolling as there is an initial platelet rolling on GpIb, and as platelets become activated, GpIIb/IIIa is upregulated and becomes a stable connection between platelets, and the adhesive surface is established. Similarly in case of leukocytes a highly extended molecule P-selectin glycoprotein ligand-1 (PSGL-1, CD162) mediates leukocyte rolling on endothelial cells (1, 2). In addition, activated platelets expressing P-selectin can be bound to leukocytes (*Figure 1*). Subsequently, leukocytes become activated, enhance Mac-1 (CD11b/18) surface expression and are subsequently fixed onto the endothelial cell surface that may terminate in transendothelial migration or may contribute to thrombotic events,

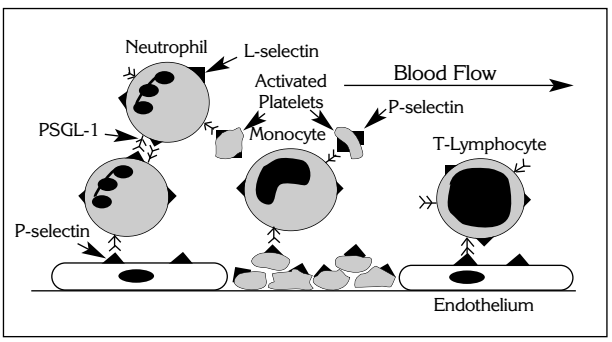

Figure 1. Interactions of P-selectin with PSGL-1 in leukocytes, platelets and endothelial cells in flowing blood

PSGL-1 is the determinant of leukocyte rolling on P- and E-selectin that are both expressed on activated endothelial cells and is also the major counterreceptor of leukocyte L-selectin. Experimental data are presented on the involvement of this receptor in acute myeloid leukaemia and haemophilia A.

PSGL-1 and acute myeloid leukaemia

In normal peripheral blood samples after labelling for PSGL-1, four populations can be differentiated on a sideward-scatter – FL1 plot (*Figure 2*). Beside neutrophils and monocytes two distinct lymphocyte populations can be identified: »PSGL-1 bright« and a»PSGL-1 dim« population. Contrary to normal neutrophils in AML blasts the expression of PSGL-1 (CD162) is significantly lower (*Figure 3*). On fluorescence and side scatter plots of AML bone marrow samples a similar distribution pattern could be observed on CD45-SSC and CD162-SSC plots. The mean fluorescence intensity (MFI) values could be established for the respective cell populations based on the CD162-SSC plots (*Figure 4*). We used precalibrated beads (QIFI-kit, DAKO) to convert the observed MFI values to actual antibody binding capacities (ABC values) that directly correspond to the PSGL-1 copy number. By using triple color staining (CD3FITC/CD19PE/CD56PE-Cy5) we found that the percentage of »PSGL-1 bright« population of the lymphocyte gate equalled with that of the CD3+ population, while the ratio of »PSGL-1 dim« population equalled with the sum of CD19+ and CD56+ lymphocytes. Thus, »PSGL-1 bright« lymphocytes are T-cells, while »PSGL-1 dim« lymphocytes are B-cells and NK-cells. The distribution of PSGL-1 on monocytes and neutrophils were unimodal and the variance in PSGL-1 labelling as determined by the variation coefficient (CV) of the MFI of PL-1 labelling was significantly smaller than that of T-cells (*Table I*). We hypothesized, that as the amount of PSGL-1 is different on mature and immature myeloid cells, low PSGL-1 expression

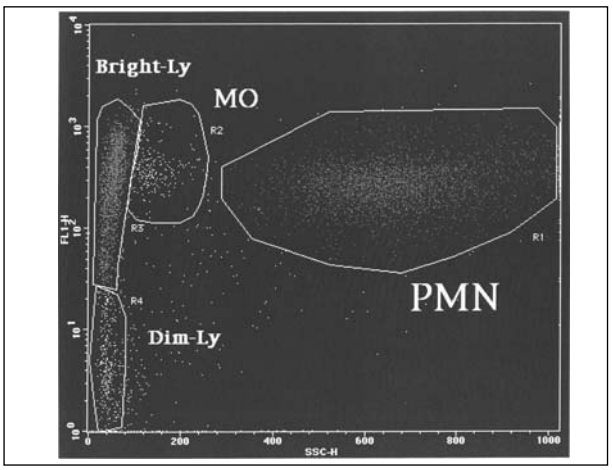

Figure 2. Fluorescence versus sideward scatter plots of normal peripheral blood leukocytes after indirect labelling with PSGL-1. Four cell populations can be identified: granulocytes (PMN), monocytes (MO), a dim and a bright PSGL-1 lymphocyte population (Bright-Ly, Dim-Ly).

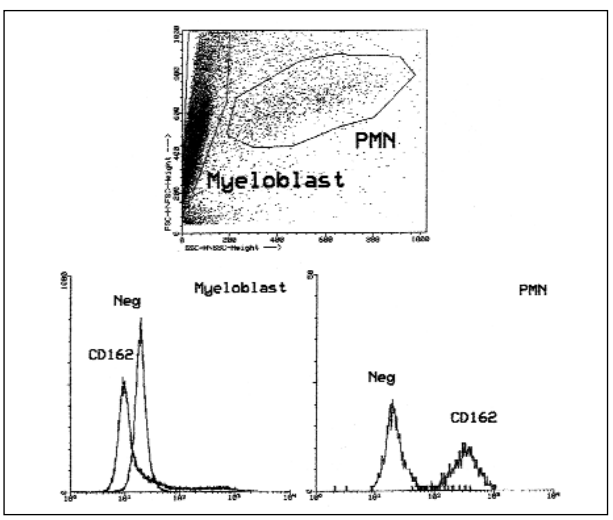

Figure 3. In some cases myeloblasts of AML samples express barely any PSGL-1, only a part of the myeloblasts are showing some labelling, while mature myeloid cells of the same sample express normal amount of PSGL-1.

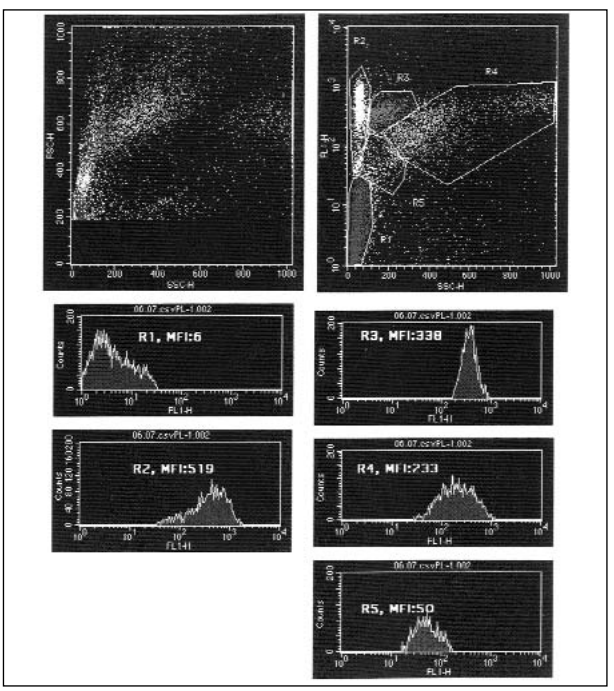

Figure 4. Fluorescence versus sideward scatter of normal bone-marrow cells. Populations than can clearly be differentiated on the fluorescence versus sideward-scatter plots largely overlap on conventional FS-SS dot plots. The histograms show the PSGL-dim (R1) and PSGL-bright (R2) lymphocyte populations and the decreasing order of PSGL expression on monocytes/monoblasts (R3), mature myeloid cells (R4) and myeloblasts (R5).

Table I Labelling characeristics of normal peripheral blood leukocytes for PSGL-1

	ABC (mean ± SD)	CV of MFI (%)
Neutrophils	26 500 ± 4 500	17
Monocytes	47 200 ± 9 900	21
T-cells	38 200 ± 26 000	68
B + NK cells	2 600 ± 1 500	58

Table II Labelling characeristics of immature myeloid cells for PSGL-1

	ABC (mean ± SD)	CV of MFI (%)
Myeloblasts (n =15)	12 000 ± 5 300	44
biphenotypic blasts (n = 2)	6 000 ± 3 100	52
Promonocytes/ Monoblasts (n = 9)	41 000 ± 20 100	49
Promyelo/myelo /metamyelo (n = 3)	19 000 ± 7 100	37

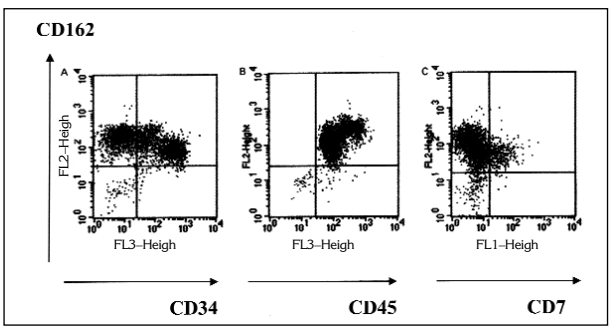

Figure 5. PSGL-1 is demonstrated in an AML M2 sample by three-colour analysis. On all plots the vertical axis (FL-2) is the labelling for PSGL-1 using PE-conjugated antiCD162 antiody. PSGL-1 dim staining is coexpressed with CD7 and CD34 positivity and with CD45dim staining.

may be a marker for acute myeloid leukaemias (AML). We analyzed 20 de novo AML samples and all were positive for CD33 and nearly all for CD13 and HLA-DR. In AML samples blast cells were identified by their low SSC and CD45 dim staining as previously described (3). This population was then backgated to the FSC-SSC plot and the PSGL-1 expression was determined. It was found that PSGL-1 (CD162) expression paralleled partly with CD45 expression and the number of CD162 on myeloblasts was significantly less than on mature myeloid cells. In samples containing mature myeloid cells and myeloblasts a clear-cut difference could be observed in PSGL-1 expression where in some cases PSGL-1 expression in malignant CD34+ cells was barely detectable. Overall, myeloblasts appearing in acute myeloid leukemia patients expressed significantly less PSGL-1 on their surfaces than mature neutrophils ($p < 0.001$). In monocytic leukaemias, however, monocytic precursors displayed CD162 expression in a fairly broad range, that did not prove to be significantly different from that of mature monocytes ($p=0.084$). Three samples were examined in the accelerated phase of chronic myeloid leukaemia enabling us to identify PSGL-1 expression on a mixture of promyelocytes, myelocytes and metamyelocytes. The average number of receptors in these cells was between myeloblasts and mature myeloid cells. The variance in PSGL-1 labelling as determined by the CV of MFI values was much larger for leukaemic cells than in mature granulocytes and monocytes. (*Table II*). When a directly conjugated monoclonal antibody (CD162-PE) was used instead of indirect labeling the most informative staining combinations were that of CD34, CD45 and CD7 (*Figure 5*). These data demonstrate a differential expression of PSGL-1 during myeloid haematopoietic development and suggests that quantification of surface PSGL-1 may help in differentiating myeloblasts from monoblasts by immunophenotyping in different AML subsets (4).

PSGL-1 and haemophilia A

It has been previously shown that elevated levels of soluble P-selectin can contribute to thrombotic processes (5, 6). Thus, we used a chimera generated by the fusion of a part of the soluble P-selectin to the Fc portion of human IgG and introduced these Psel-Ig chimeras to wild type and PSGL-1 knockout (PSGL-1 -/-) mice. In addition, we also added human Psel-Ig chimeras to human blood samples and examined the procoagulant effect by looking at the whole blood clotting time and the plasma clotting time (*Figure 6*). We found that Psel-Ig (black bars) in human blood significantly shortened both clotting times compared to a control IgG (white bars) that was dependent on PSGL-1 since the effect could be abolished by the neutralizing antibody PSG3. In the mouse *in vivo* studies, it was found that the number of microparticles (MP) is similar to the wild type and PSGL-1 -/- mice in younger animals; however, in older mice there is an upregula-

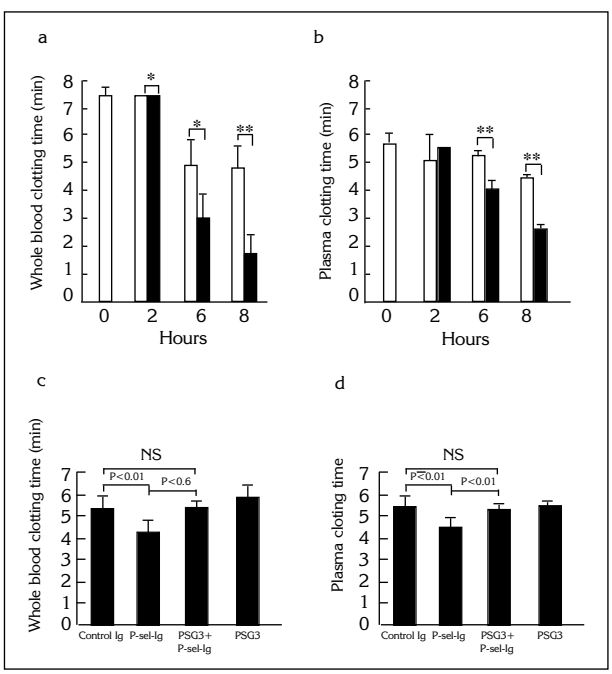

Figure 6. Whole blood and plasma clotting times from 5 normal donors were incubated at 37 °C with human P-sel Ig (black bars) or IgG (white bars). Both clotting times were reduced significantly after 6 hours (*$p<0.05$, **$p<0.005$). The lower panels show that this effect can be inhibited by a blocking antibody to PSGL-1.

Table III Role of age and PSGL-1 in MP formation

MP number PSGL-1 +/+	PSGL-1 -/-	p value
9–12 weeks 12 173 ± 2 243	14 399 ± 2746	0.54
17–20 weeks 29 404 ± 2 941	18 183 ± 1532	<0.001
After administration of Psel-Ig		
MP number/μL 833 ± 137	382 ± 101	<0.003
TF (%) 7.3 ± 1.5	2.9 ± 0.3	<0.02

Table IV Mouse *in vivo* studies

	Normal (n = 8)	Hemophiliac (n = 8)	
APTT			
IgG	23.2 ± 0.5	38.0 ± 1.5	
Psel-Ig		31.5 ± 0.7	p < 0.02
PCT			
IgG	263 ± 12	316 ± 16	
Psel-Ig		249 ± 10	p < 0.01

tion of MP number that cannot be observed in the knockout animals. The number of isolated MP was also higher in the wild type animals after Psel-Ig challenge and these MP expressed significantly more tissue factor as well (*Table II*). Since the addition of Psel-Ig was clearly procoagulant in wild type mice, we tried to exploit this phenomenon in a disorder where there is insufficient thrombin generation due to a clotting factor deficiency. Thus, Psel-Ig was administered to haemophila A mice with a factor VIII level below 1%. It was found that already 6 hours after the injection of Psel-Ig the plasma clotting time (PCT) and the activated partial clotting time (APTT) were significantly shortened (*Figure 7*). In addition, the P-sel-Ig treated haemophilia mice generated more micropartiles with elevated tissue factor concentration and showed a significant improvement in fibrin formation as evidenced by the tail bleeding assay (*Figure 8*). Similar procoagulant effect could be demonstrated in human haemophilia model where an ex vivo stimulation was carried out in 3 severely haemophilic blood samples, and the MP number and thrombin generation were followed by a chromogenic assay. In both cases the human Psel-Ig had a significant prothrombotic effect compared to the control IgG (*Table V*). This means that the missing factor VIII can be bypassed by providing another source of thrombin generation. In addition, the soluble form of PSGL-1 is now used as an effective P-selectin blocking agent at both functional and molecular level (7) that ameliorates coronary blood flow in various pathological states. Based on these studies, we can

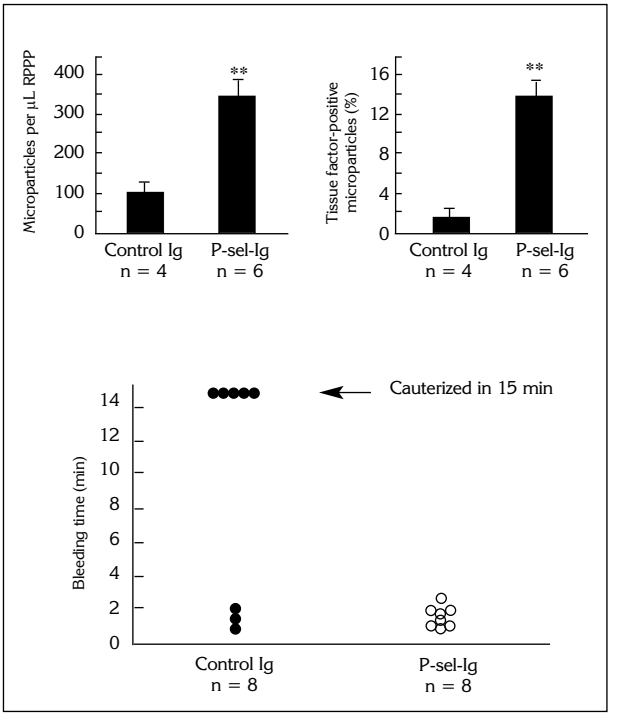

Figure 7. Psel-Ig treated hemophilia A mice generate more microparticles (MP) and the ratio of tissue factor expressing MP was also increased compared to IgG treated animals (upper panels). P-sel-Ig treated animals had significantly shorter tail bleeding time compared to IgG treated mice.

Table V Human ex vivo studies (n = 3)

Microparticle #		
IgG 22 700 ± 6 500	Psel-IG 92 700 ± 18 000	p < 0.04
TF/FX activity (mOD/min)		
IgG 4.5 ± 0.5	Psel-IG 7.9 ± 1.10	p < 0.05

establish the role of Psel-PSGL-1 interaction in the generation of procoagulant microparticles and hypothesize, that the use of Psel-Ig may be an alternative way to induce fibrin formation in coagulopathies (8).

In conclusion, we propose that PSGL-1, a heavily glycosylated mucin, may be impicated in several ways in the pathogenesis and diagnosis of various haematological disorders.

POTENCIJALNA ULOGA P-SELEKTIN GLIKOPROTEINA LIGAND-1 U HEMATOLOŠKIM OBOLJENJIMA

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Kratak sadržaj: PSGL-1 je glavni kauntereceptor od tri tipa selektina koji se izražavaju u nekoliko lekucitnih podvrsta. Rezultati koji su ovde prikazani potvrđuju da ovaj mucin ima značaja za hematološke poremećaje. Utvrđeno je u normalnoj perifernoj krvi i u uzorcima dobijenim od 20 AML pacijenta da se PSGL-1 različito izražava u pojednim leukocitnim podvrstama. Mijeloblasti koji se javljaju u akutnoj mijeloidnoj leukemiji imaju značajno manje PSGL-1 (12 000 ± 5 300) nego neutrofili ($p < 0,001$). Međutim, u monocitne leukemije količina PSGL-1 u monocitnim prekurzorima nalazi se u prilično širokoj oblasti koja se značajno ne razlikuje od one kod zrelih monocita ($p = 0,084$). Monoblasti/promonociti imaju više PSGL-1 od mijeloblasta i profil se u celosti ne preklapa. Ovo ukazuje na različito izražavanje PSGL-1 u toku miojeloidnog hemopoetskog razvoja i ukazuje da kvantifikacija površine PSGL-1 može da pomogne u razlikovanju mijeloblasta od monoblasta imunofenotipizacijom različitih AML podklasa. PSGL-1 ima takođe izvesnu ulogu u stvaranju prokoagulantnih mikročestica (MP). Kako PSGL-1 P-selektin intereaguje pri stvaranju prokoagulanog efekta ispitivana je hipoteza da uzimanje P-selektin IgG himera (Psel-Ig) koriguje krvarenje u modelu murinske hemofilije i u humanoj hemofiličnoj krvi. Dodatak Psel-Ig značajno poboljšava tendenciju krvarenja kod miševa i stvaranje MP u humanoj hemofiličnoj krvi. To znači da Psel-Ig može postati alternativni put za kontrolu tendencije krvarenja u koagulopatija.

Ključne reči: P-selektin glikoprotein ligand 1, akutna mijeloidna leukemija, hemofilija

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