

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

**Importance of P-Selectin Glycoprotein Ligand-1
in cell – cell interaction**

by Kornél Miszti-Blasius MD

Supervisor: János Kappelmayer MD, DSc



UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF KÁLMÁN LAKI
DEBRECEN, 2014

Importance of P-Selectin Glycoprotein Ligand-1 in cell –cell interaction

By Kornél Miszti-Blasius MD

Supervisor: János Kappelmayer MD, DSc

Doctoral School of Kálmán Laki, University of Debrecen

Head of the Examination Committee: György Balla MD, MHASc
Members of the Examination Committee: Kálmán Nagy MD, PhD
Gyula Reményi, MD, PhD

The Examination takes place at the library of the Department of Pediatrics, Faculty of Medicine, University of Debrecen

Debrecen, March 12, 2014, 11:00 a.m.

Head of the Defense Committee: György Balla MD, MHASc
Reviewers: Barna Vásárhelyi MD, DSc
László Rejtő MD, PhD

Members of the Defense Committee: Kálmán Nagy MD, PhD
Gyula Reményi MD, PhD

The PhD Defense takes place at the Lecture Hall of Building A of the Department of Internal Medicine, Faculty of Medicine, University of Debrecen

Debrecen, March 12, 2014, 1:00 p.m.

INTRODUCTION

The selectin family

Lectins are carbohydrate-binding proteins that have a fairly logical nomenclature. Depending on their binding requirements this family of proteins is divided into several sub-families: C-type lectins (calcium), S-type lectins (free thiol groups), P-type lectins (mannose-phosphate) I-type lectins (immunoglobulin superfamily). The selectins as a member of the C-type lectins mediate adhesion of platelets and leukocytes to the vascular surface. All selectins consist of a lectin domain followed by an epidermal growth factor like module, a varying number of short consensus repeats, a transmembrane domain and a short cytoplasmic tail.

P-selectin belongs to the selectin family (P-, L and E-selectins) and constitutes a gene cluster on mouse and human chromosome 1. The biological function of the P-selectin molecule was not evident until it was shown that it mediates the interaction of activated platelets with myeloid cells. A similar function was also observed for the endothelial P-selectin as well.

L-selectin is constitutively expressed on the leukocyte surface. However, depending on the agonist, leukocytes in the bone marrow could up- or down-regulate surface expressed L-selectin. E-selectin is expressed only on activated endothelial cells and P-selectin appears on the surface of stimulated platelets and endothelial cells. These selectins can interact with their respective glycoprotein (mucin) ligands on lymph node vessels, endothelium and leukocytes, thus selectins may catalyze multiple cellular interactions.

All three selectins can bind to P-selectin glycoprotein ligand-1 (PSGL-1) however, appropriate carbohydrate decoration and sulfation of PSGL-1 are necessary for it to reach its optimal capacity to interact with P- and L-selectins.

The structure and function of P-selectin glycoprotein ligand-1

PSGL-1 is a homodimeric mucin constitutively expressed on myeloid cells. The cytoplasmic tail of PSGL-1 communicates with intracellular proteins. Indeed the association of the PSGL-1 cytoplasmic domain with the actin cytoskeleton is crucial for rolling on P-selectin. PSGL-1 is the critical ligand for mediating leukocyte rolling on P-selectin in flow. PSGL-1 also binds to L-selectin, which *in vitro* initiates leukocyte-leukocyte interactions that amplify leukocyte accumulation on inflamed endothelial cell surfaces.

Cell adhesion molecules in different diseases

Clot formation is critical for limiting posttraumatic blood loss, but the excessive process can occlude diseased vessels leading to stroke, myocardial infarction or atherosclerosis which are the leading causes of death in the Western world. Several different molecules facilitate the activation of platelets by primary stimuli, potentially transforming a normal hemostatic response into the formation of an occlusive thrombus. The exposure of subendothelial collagen is a major contributor to such thrombotic disorders and along with other physiologically occurring platelet agonists like epinephrine, ADP and thrombin are responsible for platelet activation. Activated platelets rapidly mobilize P-selectin (CD62P, formerly described as PADGEM or GMP-140) to their surface from their alpha granules. Via the surface expressed P-selectin, platelets can form heterotypic interactions with leukocytes. The counter-receptor on the leukocyte surface is the PSGL-1. This sialomucin is the ligand for selectins which plays a central role in the formation of primary thrombus and also mediates leukocyte tethering and rolling. This cell-cell interaction is critical during coagulation, since platelets roll on activated endothelial cells, while leukocytes roll and arrest on activated platelets and endothelial cells.

Adhesion of leukocytes to platelets forms platelet-leukocyte aggregates that are deposited at the site of vascular injury.

Patients with insulin-dependent diabetes mellitus (IDDM) show a higher median plasma level of circulating P-selectin with increased P-selectin expression on platelets.

In oncology the clinical prognosis and metastatic progression of many epithelial carcinomas has been correlated with the production of tumor mucins. Metastasis is thought to involve the formation of tumor-platelet-leukocyte emboli and their interaction with the endothelium of distant organs. P-selectin that normally interacts with PSGL-1 can facilitate the metastatic seeding of mucin-producing carcinomas.

Mobilization of hematopoietic stem cells

Mobilization of leukocytes and leukocyte precursors from the bone marrow became a standard method in harvesting hematopoietic stem cells for both autologous and allogenic transplantation.

Granulocyte colony-stimulating factor (G-CSF) induces the release of a number of proteases into the bone marrow via acting on multiple cell types. Neutrophil elastase, cathepsin G as well as matrix metalloproteases —through mostly unexplored mechanisms — all contribute to this process. These proteases cleave several molecules that are thought to play an important role in hematopoietic stem cell tethering and anchorage, including L-selectin – PSGL-1 interaction, c-kit (tyrosine-protein kinase kit=CD117), VCAM-1 (vascular cell adhesion molecule-1), CXCR4 (chemokine C-X-C motif receptor 4), and SDF-1 (stromal cell-derived factor-1). These proteases cleave adhesion molecules, thus enhance the mobilization of myeloid cells and hematopoietic stem cells.

AIMS OF THE STUDY

Role of PSGL-1 molecule in thrombus formation

In the first part of our studies we induced thrombosis in mice and investigated the survival in PSGL-1 knockout and control animals. We analyzed thrombus and fibrin formation by several techniques

- We investigated the survival rate of the animals after thrombotic challenge
- Our goal was to determine the number of heterotypic aggregates and occluded vessels in PSGL-1 deficient and control mice

Role of PSGL-1 in leukocyte mobilization

The interaction between P-selectin and PSGL-1 plays an important role in the anchorage of hematopoietic stem cells to the extracellular matrix in the bone marrow. In a series of experiments we investigated the importance of the lack of PSGL-1 in the bone marrow and precursor cell mobilization.

- We examined the rate of leukocyte elimination, following an induced cytopenia, in the absence of PSGL-1
- We described the kinetics of the mobilization of leukocytes following an induced cytopenia with and without exogenous G-CSF

MATERIALS AND METHODS

Laboratory animals

C57B6/126 PSGL-1WT and KO mice were weaned at 4 weeks, maintained on a 12-hour light – 12-hour dark cycle at 21 °C, and fed water and standard rodent chow (VRF1 Charles River, Germany) ad libitum. All procedures conformed with the recommendations of the Hungarian law, the study protocol was approved by the local Ethical Committee (1/2006; 8/2011 DE MÁB) and tissue samples were obtained in accordance with current guidelines.

Models of systemic intravascular thrombosis

Wild-type (PSGL-1^{+/+}) and PSGL-1 deficient (PSGL-1^{-/-}) male mice matched for age (12-16 weeks) were anesthetized with a single dose of intraperitoneal ketamine. For „full dose” experiment 15 µg collagen (Collagen Reagent Horm, Nycomed Munich, Germany) and 3 µg epinephrine (Richter, Budapest, Hungary) was injected via tail vein and for „half dose” experiment 7.5 µg collagen and 1.5 µg epinephrine was administered. Three minutes after the injection blood samples were drawn by puncture of the retrobulbar venous plexus, the survival/exit rate was investigated up to the 30th minute after which living mice were euthanized by cervical dislocation.

Blood collection and cell counting

Mice were anesthetized with intraperitoneal ketamine (Richter, Budapest, Hungary) injection (150 mg/kg), and subsequently 200 µL blood was collected into a cup containing 40 µL ACD (from Vacutainer tube Becton Dickinson Diagnostics-Preanalytical Systems Plymouth, UK) by puncture of the

retrobulbar venous plexus with a 30 mm long glass capillary. ACD-anticoagulated whole blood was analyzed by Siemens Advia-120 hematology analyzer (Deerfield, IL).

Fibrinogen and fibrin generation

Commercially available mouse fibrinogen (Sigma, St Louis, MO) was used, while mouse fibrin was prepared by incubating 0.25 mg/mL fibrinogen in 150 mM NaCl – 10 mM TRIS at pH: 7.4 with 2 U/mL thrombin from human plasma (Sigma, St Louis, MO) at 37 °C for 15 minutes. After centrifugation (13 500 g, 4 °C, 35 minutes) the supernatant was discarded, the sediment was dissolved in SDS PAGE sample buffer (62.5 mM TRIS-HCl, 2% SDS, 10% Glycerol, 0.1% Bromphenol blue, 5% Mercaptoethanol at pH: 6.5) and the mixture was boiled for 10 minutes.

Lung fibrin determination

Lungs were harvested and then rinsed at 4 °C in extraction buffer composed of 150 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Sigma), 10 U/mL aprotinin (Richter, Hungary), 100 U/mL heparin (Merckle GmbH, Ulm, Germany), 0.1 M E-aminocaproic acid (Pannonpharma, Pécsvárad, Hungary) and 10mM Tris/HCl, pH 7.4. Samples were frozen in cryovials on the surface of liquid nitrogen and stored at -20 °C until further use. Lung tissue was thawed by immersing the cryovial in 37 °C waterbath for 5 minutes, minced, homogenized in extraction buffer at 0,5 mL buffer/100mg tissue for 10 minutes, and incubated on ice for 4 hours. The pellet, obtained after centrifugation at 16 000 g for 30minutes at 4 °C, was washed twice, resuspended in 200 µL sample buffer without bromphenol blue and mercaptoethanol.

Samples were incubated for 18 hours at 37 °C, and recentrifuged at 16 000 g for 30 minutes at room temperature. To the supernatant containing the extracted fibrin we added 5% mercaptoethanol and 0.1% bromphenol blue, samples were separated by SDS-polyacrylamide gel electrophoresis on a 7.5% gel and transferred to Immobilon P Transfer membrane (Millipore, Bedford, MA) for immunoblotting. Fibrin(ogen) was detected by reacting membranes first with a 1:2000 dilution of rabbit anti human fibrinogen (DAKO, Glostrup, Denmark), followed by horseradish peroxidase-conjugated antirabbit antibody. Bands were visualized with enhanced chemiluminescence. To quantify fibrinogen and fibrin, the optical density of bands derived from samples containing equal amounts of lung tissue were compared and normalized for actin signal by scanning densitometry.

Tissue processing for histology and immunohistochemistry

For comparative light microscopy analysis, tissue samples obtained from both animal groups were fixed with Saint-Marie solution, embedded in paraffin, and stained with hematoxylin-eosin or Masson trichrome. For immunohistochemical analysis 5 µm sections were deparaffinized, rehydrated followed by antigen unmasking in pressure cooker (120 °C, 3 mins) using antigen retrieval solution (pH 6.0, DAKO, Glostrup, Denmark). Sections were then treated with 1% H₂O₂ in methanol for 30 minutes at room temperature to block endogenous peroxidase. Following pretreatment with 3% nonfat dry milk in background reducing solution (DAKO), sections were incubated with mAb350 (1:40 dilution) which detects only cross linked fibrin at room temperature for 1 hour. After washing with PBS, visualisation was carried out by the use of biotin-free EnVision detection kit (DAKO).

To check the specificity of immunostaining, negative controls were included where no antibody or irrelevant control monoclonal antibody (DAKO) was used in place of the primary antibody that did not show labeling. To quantitate the accumulation of thrombi in the lung tissue sections, the number of occluded vessels in 12 randomly selected high magnification microscopic fields from 4 mice in each treatment group were analyzed.

Platelet-leukocyte aggregate formation

Fifty μl of ACD (Acid Citrate Dextrose)-anticoagulated mouse blood was simultaneously labeled by rat anti-mouse CD14 PE (phycoerythrin) and rat anti-mouse CD41 FITC (fluorescein-isothiocyanate) for 15 minutes at room temperature. Leukocyte subsets were identified by the SSC-FL2 dot plots and heterotypic aggregates were measured by the CD41 fluorescence values in respective leukocyte gates. Thrombin receptor agonist peptide (TRAP) stimulation at 37 °C for 15 minutes was used to enhance heterotypic aggregate formation using 10 $\mu\text{mol/L}$ of the agonist. For blocking experiments, blood samples were preincubated with 2 $\mu\text{g/mL}$ rat anti- mouse PSGL-1 or rat IgG as negative control for 15 minutes.

Induced cytopenia and G-CSF treatment

Wild-type and knockout male mice matched for age (12–16 weeks) were anesthetized with Halothane (Sigma-Aldrich, St. Louis, MO, USA) and a single dose of cyclophosphamide (Endoxan, Baxter, IL, USA) was administered intraperitoneally (250 mg/kg) to reach cytopenia. G-CSF (Neupogen, Amgen, Thousand Oaks, CA, USA) treatment was applied twice a day at 7.8 $\mu\text{g/kg}$ body weight subcutaneously for 4 days.

Flow cytometric analysis of stem cells

For flow cytometric analysis of mouse peripheral blood samples three-color stainings were applied by using rat anti-mouse CD34/CD162/CD45 and CD34/CD117/CD45 combinations. R-Phycoerythrin (R-PE)-conjugated rat anti-mouse CD162 (PSGL-1), R-Phycoerythrin (R-PE)-conjugated rat anti-mouse CD117, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD34 and Peridinin-chlorophyll proteins (PerCP)-conjugated CD45 monoclonal antibody were from BD Pharmingen (Becton Dickinson, Franklin Lakes, NJ, USA). Flow cytometric analyses were performed on a FACSCalibur flow cytometer (50 000 events were collected) and data were analysed by the CELLQuest 3.2 software (Becton Dickinson, Franklin Lakes, NJ, USA).

Tissue processing for quantitative immunohistological analysis of stem cells

After harvesting the mice, femurs were removed for histological processing. After fixation, tissue samples were placed into 10 % EDTA; pH 7.2 (Solon, Ohio) for approximately 2 weeks. Decalcified and dehydrated tissue samples were embedded into wax at 54 °C and 5–6 µm thick longitudinal sections were cut perpendicular to the long axis of femur. Paraffin sections were placed on gelatin-coated glass slides and left to dry overnight at 37 °C. To identify CD34, standard immunohistochemical (IHC) reaction and ABC techniques were performed. Before IHC staining, sections were deparaffinised in xylene and hydrated through graded alcohols to water. After rehydration, sections (5–7 µm) were subjected to 0.5 % H₂O₂ in absolute methanol to block the endogenous peroxidase activity. After PBS washing, slides were rinsed in 2% BSA in PBS for 30 min at 37 °C in a humid chamber to minimize nonspecific staining. The sections were incubated for 1 h at 37 °C with the rabbit anti-CD34 antibody (Chemicon, Temecula, CA, USA) in PBS (1:75, containing 0.1 % BSA).

After a brief wash in PBS, the samples were treated for 1 h at room temperature with biotin-conjugated anti-rabbit secondary antibody (Vectastain Elite ABC Kit; Vector, Burlingame, CA, USA) diluted in 1:200 PBS containing 0.1% BSA. Then, the slides were treated for 30 min at room temperature with streptavidin–biotin peroxidase complex (Vectastain Elite ABC Kit, Vector). After rinsing in PBS, the reaction was visualized with Vector peroxidase substrate Kit.

CFU-GM colony formation assay

Mononuclear cells were separated both from peripheral blood and bone marrow and were grown in special soft gel cultures. After exterminating the mice, bone marrow was obtained by expulsion from their femurs under sterile conditions and single cell suspensions were prepared in McCoy's 5A medium. The mononuclear cell fraction, from both peripheral blood and bone marrow, was separated by density gradient centrifugation using Ficoll-Paque™ Plus density gradient media (specific gravity, 1.077 g/mL). Separated mononuclear cells from peripheral blood and bone marrow were plated in Petri dishes at a density of 6×10^5 and 1×10^5 cells per dish, respectively and cells were grown in McCoy's 5A modified medium (Sigma-Aldrich, Budapest, Hungary) supplemented with amino acids, Na-pyruvate, NaHCO_3 , antibiotics (streptomycin, penicillin) and 20 % fetal bovine serum. In the presence of WEHI-3B conditioned medium, a crude source of interleukin-3 and granulocyte-colony stimulating factor, granulocyte–macrophage progenitor cells (colony forming unit-granulocyte–macrophage [CFU-GM]) form colonies in special soft gel cultures. Methylcellulose (Methocel, 3.000–5.000 CP; Fluka, Buchs, Switzerland) at 1.2 % was used as the support matrix for these semi-solid cultures. Cultures were grown in duplicates for 7 days at 37 °C at 100 % relative humidity in an atmosphere containing 5 % CO_2 .

Colonies were defined as groups of at least 50 cells and were counted under a stereomicroscope (Olympus, Hamburg, Germany) at the end of the incubation period.

Statistical analyses

For comparison of exit rates of challenged mice results were evaluated by the Chi-square test. One Way ANOVA was used for the evaluation of thrombosed vessels. Cell counts were compared by Student's t-test. In the cell mobilization study data showing Gaussian approximation Student's t -test was used. In case of nonparametric distribution results were evaluated by Mann–Whitney test using the GraphPad Prism 4.0 software (La Jolla, CA, USA).

RESULTS

The effect of thrombotic challenges on survival rate

The survival rate of mice was evaluated in 87 animals, 30 minutes after the thrombotic challenge. Upon administration of the full dose collagen and epinephrine (15 μ g and 3 μ g, respectively) 8 mice out of 13 (62%) died from the PSGL-1^{+/+} group and 7 of the 15 mice (47%) from the PSGL-1^{-/-} strain within 30 minutes (p=0.476). Reducing the concentration of the thrombogenic agents by 50% (7.5 μ g collagen and 1.5 μ g epinephrine) resulted in a considerable difference in survival rates since 15 animals of 30 (50%) in the PSGL-1^{+/+} group died while only 3 out of 29 (10%) in the PSGL-1^{-/-} group (p=0.002).

Cell counts in survived animals

Collagen and epinephrine administration resulted in a dose dependent thrombocytopenia. Platelet count decreased significantly ($p < 0.0001$) in both groups after applying the full dose challenge and the difference between the two strains was not significant ($p = 0.261$). On the contrary, after administrating half dose of the prothrombotic agents, the platelet count reduction was significantly less in the PSGL-1^{-/-} mice ($p = 0.0325$). The decrease in leukocyte count was similar in the two groups and was independent from the applied dose of collagen and epinephrine. Since peripheral blood neutrophil count is significantly elevated in the absence of PSGL-1 mice, we investigated the changes in absolute neutrophil counts before and after the thrombotic challenge in 6 and 9 animals in PSGL-1^{+/+} and PSGL-1^{-/-} strains respectively. Before the thrombotic challenge the absolute neutrophil counts were 3-times higher in knockout animals, however the values decreased in a similar manner in both strains.

Thrombin generation and fibrin deposition

Fibrin deposition in lungs was assayed by Western blot analysis of lung fibrin extracts using the anti-human polyclonal antibody – that crossreacts with mouse proteins – to detect both fibrin and fibrinogen. Our results showed that after the injection of thrombotic agents more fibrin deposits in the lungs of PSGL-1^{+/+} mice than in those of PSGL-1^{-/-} mice.

Morphological analysis of thrombi

Our immunohistochemical (IHC) analysis demonstrated that several thrombi were formed in the lung vasculature after thrombotic challenge. First, sections were analysed after Masson trichrome staining to identify thrombosed vessels.

Their number was more abundant in wild-type animals and these thrombi also tended to occlude large vessels. In the PSGL-1^{-/-} animals less thrombi has been observed and they were occlusive in large vessels. Fibrin could be identified immunohistochemically in these thrombi on sections derived from both strains by using mAb350 antibody that detects only fibrin but not fibrinogen. The percent of the thrombosed vessels was found to be higher in wild-type animals.

Platelet-leukocyte aggregate formation

Neutrophils from wild-type mice showed high values of CD41 (platelet glycoprotein IIb) fluorescence that increased upon stimulation with TRAP, as platelets adhered to myeloid cells. This effect was completely blocked by the anti-PSGL-1 antibody that also prevented TRAP induced elevation of CD41 fluorescence. Similar results were obtained for monocytes. No changes in lymphocyte associated fluorescence were observed. PSGL-1^{-/-} mice were negative for CD41-related staining for all cell types.

Leukocyte elimination after induced cytopenia

Leukocyte count decreased by 80–90% in both mouse strains after cyclophosphamide administration however, the elimination kinetics differed in the two strains. In PSGL-1^{+/+} animals, the absolute counts of all cell types decreased while in PSGL-1^{-/-} mice the neutrophil and monocyte absolute numbers were elevated on day 1. It started to decline on day 2 and only returned to baseline value. Both strains showed the lowest values on day 3. Knockout mice displayed a significantly larger rebound effect on day 6 peaking at three times the baseline values.

Spontaneous leukocyte mobilization

When this rebound effect was analyzed in detail in seven wild-type and seven knockout mice it was observed that in PSGL-1^{-/-} animals at day 7, both neutrophils and monocytes displayed significantly higher counts. Seven days after the injection of cyclophosphamide the neutrophil counts were 3.0 G/L in wild-type and 12.5 G/L in knockout animals and decreased considerably by day 11. The monocyte count decrease could be observed only in the PSGL-1^{+/+} strain after the seventh day but it also returned to normal in the PSGL-1^{-/-} mice when recovery was followed up to day 16. Contrary to these cells, eosinophilic granulocytes displayed a delayed response and were nearly undetectable at day 7 and only became elevated by day 11 being much higher in knockout animals too. Furthermore at day 7, morphologically atypical neutrophils (precursor cells) also appeared that were barely detectable before the induced cytopenia.

G-CSF induced leukocyte mobilization

In another series of experiments, mice received 7.8 µg/kg body weight exogenous G-CSF during 4 days (twice a day). Four hours after the last injection of G-CSF, retro-orbital blood samples were drawn and the WBC count was determined.

In 15 wild-type mice and in 15 knockout mice the mean neutrophil counts were 28.3 and 47.7 G/l, respectively, while the mean monocyte counts were 2.0 and 4.1 G/l, respectively. Neutrophil, monocyte and eosinophil absolute cell counts were significantly elevated (p <0.05 to p <0.001) in PSGL-1^{-/-} compared to PSGL-1^{+/+} mice. The absolute number of neutrophils and monocytes but not eosinophils and atypical cells exceeded that of the results of the previous experiments without exogenous G-CSF treatment.

CFU-GM analysis in the peripheral blood and bone marrow

Based on the results of the colony-forming assays, we found a continuously higher CFU-GM count in the peripheral blood in PSGL-1^{-/-} mice even after returning to the likely baseline values on day 5. They released four times as much CFU-GM into the circulation than PSGL-1^{+/+} mice. A considerable reduction was seen by day 5, but the number of the mobilized CFU-GM remained significantly higher ($p < 0.01$) compared to wild-type mice. In the bone marrow, cellularity was investigated as total mononucleated cell number, that decreased on the first days after cyclophosphamide-induced damage as a consequence of the death of proliferating progenitor cells. The cellularity mirrored the whole hemopoiesis; however, the CFU-GM participated in the regeneration of cellularity of bone marrow that paralleled with expansion of the CFU-GM pool. In the bone marrow, the number of CFU-GM colonies per 10^5 mononuclear cells increased in parallel in both PSGL-1^{-/-} and PSGL-1^{+/+} mice after induced cytopenia, but their number became significantly higher in PSGL-1^{-/-} animals on day 3 ($p < 0.05$). As a result, the expansion of the CFU-GM population was significantly higher in PSGL-1^{-/-} than in wild-type mice on days 4 and 5 in the bone marrow.

Stem cell analysis in the peripheral blood and the bone marrow

These changes were in agreement with the peripheral blood CD34⁺/CD117⁺ mouse stem cell numbers, since on day 5 these numbers were also significantly higher in PSGL-1^{-/-} mice compared to wild-type animals as detected by two-color flow cytometry. By day 7, however, the stem cell numbers considerably increased in the peripheral blood in the PSGL-1^{+/+} mice, which could also be verified in the bone marrow by immunohistochemistry.

DISCUSSION

The lack of PSGL-1 is protective in a thrombosis model

Interactions between leukocytes, platelets and the endothelium play an important role in thrombosis. Platelet reactions are mediated by specific cell adhesion molecules that generate homotypic and heterotypic adhesive interactions with other platelets, or leukocytes, endothelial cells and the extracellular matrix. Earlier studies focused mainly on PSGL-1 mediated cell rolling and considerably less is known about the function of PSGL-1 in platelet aggregation and thrombosis. Although tremendous progress has been made to study the mechanism for leukocyte activation and transmigration across an injured vascular surface, limited data are available about the role of selectin-ligand interaction during thrombosis. The lack of P-selectin has previously been shown to attenuate thrombus formation and cell rolling but this has not been proven for PSGL-1 in case of a systemic thrombosis model. To have a better insight into this molecular mechanism, we applied the collagen/epinephrine challenge method. By using the conventionally applied dose, we found that the lack of PSGL-1 showed a non-significant protective effect in mice.

Introducing 50% of the thrombotic stimuli, this protection was more evident and highly significant differences were observed in survival rates. With the smaller dose only 10% of the PSGL-1^{-/-} mice died in 30 minutes while 50% of the wild-type animals suffered fatal thrombosis. After the large dose stimulus 62% of the wild-type and 47% of the PSGL-1^{-/-} mice died, these results suggest that the higher dose masks the protective effect of PSGL-1. The difference in platelet count decrease was not significant between the two strains studied applying full dose challenge, however reducing the dose to half, the platelet count reduction was significantly attenuated in the PSGL-1^{-/-} mice.

These data provide evidence that the absence of this ligand may play an important role during thrombus formation and platelet-leukocyte interaction. We found a significant and similar type of decrease in neutrophil percentage in both wild-type and PSGL-1^{-/-} mice and also confirmed the higher percentage of myeloid cells. Platelets were activated directly with collagen plus epinephrine via tail vein to initiate thrombus formation. In our preliminary experiments we observed that the microthrombi deposition was mostly evident in the lung since that is the organ that possesses numerous small arteries, capillaries and it also has a considerable blood flow. By immunoblotting, more fibrin was detectable in wild-type mice and this result was further confirmed by microscopic evaluations. In hematoxylin-eosin stained, as well as Masson-stained sections and sections applying monoclonal antibody staining against cross-linked fibrin, more thrombi were detectable in PSGL-1^{+/+} mice and these thrombi were occlusive or near occlusive. In contrast to this phenomenon, in PSGL-1-null mice the lumen of the blood vessels was partially obstructed but not occluded and the percent of thrombosed vessels was significantly lower in PSGL-1^{-/-} mice. We suggest, that the lower fibrin deposition in PSGL-1-null mice is related to the formation of less thrombi in these animals. Platelets and leukocytes could be observed in co-localization in thrombi on sections derived from both strains.

To provide further evidence to the knockout approach we could prove the role of PSGL-1 in thrombotic processes in wild-type animals by using the heterotypic aggregate model, where blocking antibody to PSGL-1 completely abolished the platelet binding of neutrophils. Genetically modified mice are useful to develop different models or understanding, preventing and treating human diseases. Although mice have higher platelet count, different signal transduction and different vascular rheology, the thrombosis model described above can provide helpful data about human thrombus generation and can help in development of drugs that block P-selectin-PSGL-1 interaction in arterial thrombosis.

The expression of PSGL-1 is widespread in the body and aside from the myeloid, lymphoid and dendritic cell lineage endothelial cells and platelets have also been shown to be a source of this protein. Our findings indicate, that the lack of PSGL-1 can prevent experimental thrombosis. This effect may be related to the widespread expression of this mucin and to the multitude of intercellular reactions that are mediated via PSGL-1. In human practice monoclonal antibodies against P-selectin, PSGL-1, L-selectin and E-selectin can be applied to inhibit leukocyte rolling and protect patients from myocardial reperfusion injury. Platelet-leukocyte interactions and the subsequent endothelium activation are important factors in thrombus formation and these clinical data are in line with our experimental observations and suggest the beneficial effect of PSGL-1 inhibition during atherothrombotic disorders.

Importance of PSGL-1 in stem cell mobilization

Cellular release from the bone marrow is orchestrated mostly by receptor pair interactions, which are all required to maintain adequate release of precursor and mature myeloid cells upon appropriate signals. These interactions have been examined in detail however, it is not fully clear as to what extent these receptorial interactions are redundant, or whether these bindings are all important for a timely cellular release. Therefore in the mouse model experiments presented here, our aim was to provide data on the effect of PSGL-1 in mediating cellular anchorage. Lack of PSGL-1 may impair cellular interactions at two sites. On the one hand, the receptor is expressed in the extracellular matrix, and this mucin binds to the leukocyte L-selectin facilitating adhesion of hematopoietic stem and progenitor cells. Furthermore, this dimeric mucin is crucial in the extravasation of myeloid cells in the peripheral blood. Activated endothelial cells express P-selectin and E-selectin that are the primary determinants in short-lived interactions with rolling leukocytes.

Leukocytes establish this connection via the constitutively expressed PSGL-1 molecule. Here, for the first time, we provided experimental evidence that in the absence of PSGL-1, neutrophil and monocyte extravasation is considerably slower and thus are retained in the circulation for prolonged periods when released from the bone marrow after cyclophosphamide induced cytopenia. The release of different myeloid cell types from the bone marrow was variable. Neutrophils and monocytes were released with quite similar kinetic pattern both with or without the use of exogenous G-CSF. The ratio of myeloid cell increase in both set of experiments was similar in the two strains, but this resulted in significantly higher absolute myeloid cell numbers in the PSGL-1^{-/-} mice. The only difference between the two set of experiments was that without exogenous G-CSF, monocytes reached their peak count on day 11, while with exogenous G-CSF their number peaked already at day 7 similarly to neutrophils. This may be related to the slower release of monocytes from the bone marrow or much rather for their prolonged presence in the circulation in the absence of PSGL-1 but these differences disappeared by day 16. The release of eosinophils, however was delayed in both series of experiments and—unlike in case of neutrophils and monocytes — their absolute number was not augmented by the addition of exogenous G-CSF.

Since eosinophils reside primarily in the gastrointestinal mucosa and normally constitute only 1–4 % of blood nucleated cells, their kinetic of appearance and disappearance may be much different than that of neutrophils. In addition, there are numerous factors that may contribute to this phenomenon. G-CSF does not act directly on eosinophils however, their release may be induced by several other cytokines (e.g., IL-2, IL-3, IL-5 and IL-17), thus resulting in a delayed indirect stimulatory effect. It has also been observed that fewer eosinophils than neutrophils accumulate at both P- and E-selectin surfaces. Furthermore, eosinophils are also known to form fewer leukocyte–leukocyte interactions than neutrophils.

For the proper interpretation of these findings, it is important to note that according to previous studies G-CSF reduces surface PSGL-1 expression and adhesion to P-selectin in vitro. Thus, it is quite likely that in the G-CSF treated wild-type animals not only more cells were mobilized from the bone marrow but cells retained somewhat longer in the circulation due to the partial loss of their surface PSGL-1; hence, reduced tethering and rolling could occur. Indeed, we have also witnessed a decrease in PSGL-1 expression in both human and mouse samples. All myeloid cell types attenuated CD162 expression upon incubation with G-CSF, while PSGL-1 positive lymphocytes displayed no change. Extravasation may also be hampered in both strains due to the well-known effect of G-CSF to downregulate L-selectin — an important mediator of cell adhesion. In addition to its role on the selectin–selectin ligand interaction, G-CSF also causes SDF-1 decrease on the surface of osteoblasts. Atypical neutrophilic precursor cells were analyzed and their number was found to be significantly elevated in knockout mice compared to wild-type animals. The number of these cells however was not different in the absence or presence of exogenous G-CSF. The release of precursor cells is of primary importance in G-CSF treatment thus, we utilized colony forming assays in blood and bone marrow samples and similarly, we identified stem cells at these locations by flow cytometry and immunohistochemistry.

The majority of colony forming cells consisted of lineage-restricted colonies. In our assay we verified a much higher number of CFU-GM colonies in the peripheral blood throughout the investigated induced cytopenia period. In the bone marrow of the femur an elevation was observed in the number of colonies during the induced cytopenia but with a different kinetics in the two strains. PSGL-1^{-/-} mice displayed significantly more CFU-GM colonies in the femur. Although colony-forming assays are informative about the progenitor cell content, the colony forming cells can not be equaled to hemopoietic stem cells.

Nevertheless, the bone marrow CFU-GM content paralleled with the number of stem cells as evaluated by double positivity for CD34 and CD117. In human studies stem cells are identified by their CD45⁺/CD34⁺ staining that excludes circulating endothelial cells. In mice it should be noted, that there may be a fluctuation in staining since the CD34 expression is reversible and so the CD117⁺/CD34⁺ double positive event count more reliably identifies stem cells. At the peak of the mobilization and also in agreement with previous studies, we detected significantly elevated amounts of CD34/CD117 positive progenitors in wild-type but not in PSGL-1 deficient mice. These data were in agreement with the immunomorphological findings in the bone marrow where more CD34 positivity was observed in wild-type animals both in the untreated ones and also in the ones after induced cytopenia. It has been known for quite some time that PSGL-1 not only mediates the attachment of mature leukocytes to P-selectin, but is also the sole receptor of Pselectin on primitive CD34⁺ HPCs. These very immature cells contain considerably less copies of other adhesive receptors (e.g., CD44 and CD49d) and are probably more prone to be released in the absence of PSGL-1. Blasts and stem cells, on the other hand, express significantly more of these adhesive receptors, which may result in different kinetic of stem cell release when the PSGL-1-selectin anchor is missing. It should be noted that the dose of G-CSF was lower in our experiments than described previously for mouse studies and was in the range of 5–10 µg/kg that were utilized in previous human studies. In our experiments both with and without exogenous G-CSF, the elevation in neutrophil and monocyte counts in PSGL-1^{-/-} mice compared to wild-type animals was striking. Thus, in the lack of PSGL-1, G-CSF is more potent in mobilizing a larger number of myeloid cells and particularly myeloid precursors. This raises the possibility of applying PSGL-1 blockade for the enhancement of bone-marrow release of myeloid cells that may also be retained longer in peripheral blood.

SUMMARY

Interactions between leukocytes, platelets and the endothelium influence inflammation and atherosclerosis. Platelets can be activated by specific cell adhesion molecules or soluble mediators and play an important role in the initial phase of inflammation and endothelial cell damage. This process can lead to formation of atherosclerotic plaque and later thrombosis.

Myocardial infarction, ischemic stroke and deep venous thrombosis are the leading causes of death in the modern world, a common phenomenon in these disorders is that P-selectin appears on the surface of activated platelets that crossreacts with leukocytes via PSGL-1 and thus, heterotypic aggregates are formed. Leukocytes adhere to the endothelial cells via the same ligand.

Our findings indicated, that mice lacking PSGL-1 were more resistant to the thrombotic challenge elicited with collagen and epinephrine. In these PSGL-1^{-/-} mice less thrombi were detected by two different microscopic methods. In addition, fibrin deposition was considerably attenuated in the PSGL-1^{-/-} animals. These effects were due to the absence of P-selectin PSGL-1 interaction, since the blocking of this binding eliminated the effect as observed by the changes in the number of heterotypic aggregates.

Interaction between P-selectin and PSGL-1 may also mediate myeloid cell mobilization from the bone marrow and transendothelial migration of leukocytes. In clinical practice, cell mobilizing agents (G-CSF, GM-CSF, AMD-3100) are commonly used. In a series of experiments we demonstrated in mice lacking PSGL-1 that the mobilization of myeloid progenitor cells is faster than in wild-type animals. Furthermore, a significantly elevated number of colony forming unit granulocyte-macrophage (CFU-GM) was detected also in the femurs of PSGL-1^{-/-} mice, after cyclophosphamide treatment and these values paralleled with the elevation of CD34⁺/CD117⁺ stem cell counts in the peripheral blood.

Our data suggest, that in the absence of PSGL-1, G-CSF was more potent in elevating absolute myeloid cell numbers by acting on cell release from the bone marrow, maturation from circulating precursor cells in the peripheral blood and promoting prolonged presence in the circulation.

SUPPORTS

This research was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4. A/2-11-1-2012-0001 ‘National Excellence Program’.



SZÉCHENYI TERV



Register Number: DEENKÉTK/27/2014.

Item Number:

Subject: Ph.D. List of Publications

Candidate: Kornél Miszti-Blasius

Neptun ID: AJ4XM4

Doctoral School: Kálmán Laki Doctoral School

Mtmt ID: 10034630

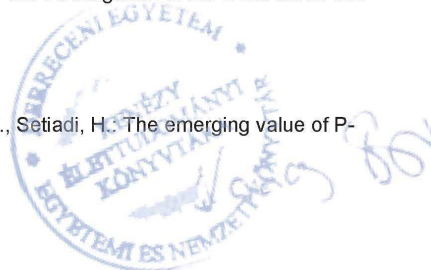
List of publications related to the dissertation

1. **Miszti-Blasius, K.**, Felszeghy, S., Kiss, C., Benkő, I., Géresi, K., Megyeri, A., Hevessy, Z., Kappelmayer, J.: P-selectin glycoprotein ligand-1 deficiency augments G-CSF induced myeloid cell mobilization.
Naunyn Schmiedebergs Arch. Pharmacol. 387 (2), 109-118, 2014.
DOI: <http://dx.doi.org/10.1007/s00210-013-0913-9>
IF:2.147 (2012)
2. Nagy, B., **Miszti-Blasius, K.**, Kerényi, A., Clemetson, K.J., Kappelmayer, J.: Potential therapeutic targeting of platelet-mediated cellular interactions in atherosclerosis and inflammation.
Curr. Med. Chem. 19 (4), 518-531, 2012.
DOI: <http://dx.doi.org/10.2174/092986712798918770>
IF:4.07
3. **Miszti-Blasius, K.**, Beke Debreceni, I., Felszeghy, S., Dezső, B., Kappelmayer, J.: Lack of P-selectin glycoprotein ligand-1 protects mice from thrombosis after collagen/epinephrine challenge.
Thromb. Res. 127 (3), 228-234, 2011.
DOI: <http://dx.doi.org/10.1016/j.thromres.2010.11.022>
IF:2.44



List of other publications

4. Kappelmayer J., **Miszi-Blasius K.**: Laboratóriumi vizsgálatok.
In: Urológia 2. átdolg. kiad. Szerk.: Tóth Csaba, Medicina, Budapest, 86-97, 2014.
5. Horváth K., **Miszi-Blasius K.**, Gyurina K., Szegedi I., Kiss C.: Herediter sphaerocytosis - A széles spektrumú betegség.
Gyermekgyógyászat. 64 (5), 217-220, 2013.
6. **Miszi-Blasius K.**, Esze R., Szegedi I., Kiss C., Kappelmayer J.: Diagnosztikai lehetőségek örökletes hemolitikus anémiákban.
Gyermekgyógyászat. 64 (5), 213-214, 2013.
7. Palicz, Z., Jenes, Á., Gáll, T., **Miszi-Blasius, K.**, Kollár, S., Kovács, I., Emri, M., Márián, T., Leiter, É., Pócsi, I., Csósz, É., Kalló, G., Hegedűs, C., Virág, L., Csernoch, L., Szentesi, P.: In vivo application of a small molecular weight antifungal protein of *Penicillium chrysogenum* (PAF).
Toxicol. Appl. Pharmacol. 269 (1), 8-16, 2013.
DOI: <http://dx.doi.org/10.1016/j.taap.2013.02.014>
IF:3.975 (2012)
8. Kotán, R., Németh, N., Kiss, F., Pósn, J., **Miszi-Blasius, K.**, Tóth, L., Furka, I., Mikó, I., Sápy, P., Szentkereszty, Z.: Micro-rheological changes during experimental acute pancreatitis in the rat.
Clin. Hemorheol. Microcirc. 51 (4), 255-264, 2012.
DOI: <http://dx.doi.org/10.3233/CH-2012-1531>
9. Németh, N., Kiss, F., Magyar, Z., **Miszi-Blasius, K.**, Furka, I.: Following-up hemorheological consequences of gonadectomy in male and female rats.
Clin. Hemorheol. Microcirc. 50 (4), 231-243, 2012.
DOI: <http://dx.doi.org/10.3233/CH-2011-1430>
10. **Miszi-Blasius K.**, Veszprémi A., Kissné S.V., Kappelmayer J.: A liquor fehérvérsejtszám és sejtösszetétel vizsgálatának algoritmusai =Algorithm for the investigation of the white blood cell count and cell differentiation in cerebrospinal fluid.
Klin. Kísér. Lab. Med. 33 (2), 11-16, 2008.
11. Kappelmayer, J., Nagy, B., **Miszi-Blasius, K.**, Hevessy, Z., Setiadi, H.: The emerging value of P-selectin as a disease marker.
Clin. Chem. Lab. Med. 42 (5), 475-486, 2004.
DOI: <http://dx.doi.org/10.1515/CCLM.2004.082>
IF:1.685



12. Nagy B., Veszprémi A., Kiss F., **Miszi-Blasius K.**, Kappelmayer J.: Thrombocyták aktiváltsági állapotának vizsgálata áramlási citometriai módszerekkel =Detection platelet activation status by flow cytometric methods.
Klin. Kísér. Lab. Med. 30, 46-53, 2003.

Total IF of journals (all publications): 14.317

Total IF of journals (publications related to the dissertation): 8.657

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

07 February, 2014

