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Modulation of cell surface receptor expression in platelets and leukocytes

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INTRODUCTION AND REVIEW OF LITERATURE

In the 19th century, Max Schultze and Giulio Bizzozero published the important role of anucleated platelets in hemostasis in case of vascular injury. Platelets travel with the bloodstream to the site of injury and there they adhere to the vessel wall and to each other and then the thrombus reaches the size with which it obstructs the lumen of the vessel, thus preventing further blood loss. Subsequent studies have described several components and mechanisms which sensitize platelets to stimuli, as well as identified several mechanisms by which uncontrolled activation of platelets compromises the integrity of the vascular system. Nowadays, leading causes of death are cardiovascular and thrombotic diseases, platelet activation and their interactions play a fundamental role in the pathogenesis of these diseases. The difference between the onset of physiological hemostasis and pathological thrombotic processes is very small, so constant control of the process is required. Numerous studies have reported that platelets play a role not only in hemostasis but also in immune and inflammatory processes, contributing to angiogenic and developmental processes, direct destruction of microorganisms and the development of tumor metastases.

In our studies, we investigated the activation of platelets through their immune receptors, which may contribute to the development of cardiovascular and thrombotic diseases. Basically, the studies were carried out with two approaches (i) on the one hand we performed in vitro experiments (ii) on the other hand we examined ex vivo samples from different diseases that are relevant to our studies. In these experiments, we not only characterized the activation state of the cells by determining partly cellular and partly soluble markers, but also examined the pathomechanisms underlying the observed differences.

Cell surface receptors of platelets

Platelets express several cell surface receptors, among them adhesion receptors, receptors that regulate thrombus formation and purinergic receptors that primarily act as sensors in hemostasis processes. While receptors that recognize patterns, antigens or chemokines and bind sialic acid play a major role in immunity. The best characterized function of platelets is their role in hemostasis, so it is not surprising that most publications deal with the role of receptors in hemostatic processes. Some receptors do not just perform signal transduction in cells but they also often play a dual role as adhesion molecules and also determine the reactivity of platelets “phenotype”.

Integrins are a major class of adhesion and signaling molecules, most of them undergo a conformational change during platelet activation to increase their affinity for the ligand. When the vessel wall is damaged in the first step platelet α2β1 integrin (GPIa-IIa) binds to collagen, and then platelet adhesion is increased through the glycoprotein VI (GPVI) receptor and platelets become activated. At high shear stress, neither α2β1 nor GPVI is sufficient for adhesion, GPIb receptor of platelets and plasma von Willebrand factor (vWF) are required for binding of platelets to collagen. αIIbβ3 (GPIIb-IIIa) belongs to another family of integrins, and it is expressed only on platelets. Upon activation it undergoes a conformational change and as a result the RGD binding site is expressed on the surface and binds fibrinogen with high affinity. These changes are essential in platelet adhesion, aggregation and also in clot retraction. The second most abundant adhesion receptor on platelets is the GPIb-IX-V complex, which belongs to the family of leucine-rich repeats and plays an essential role in platelet adhesion at high shear stress. During collagen-induced platelet activation via the cell adhesion GPVI receptor kinases of the Sarcoma family (SFKs), namely Lyn and Fyn kinases, phosphorylate the ITAM region of the FcRγ chain that triggers phosphorylation of Syk tyrosine kinase. This step initiates a downstream signaling cascade involving the activation of phospholipase Cγ2 (PLCγ2) and platelet activation occurs, during an inside-out process GPIIb/IIIa is activated in which SFKs, Lyn, Fyn and Src kinases also play a significant role.

Upon platelet activation, P-selectin (CD62P) adhesion receptor is member of the C-type lectin receptor family, it is released from the α-granule to the platelet surface and link to a major co-receptor, PSGL-1.

PAR1 and PAR4 are expressed by human platelets and belong to protease activated receptors (PARs). It is a group of thrombus formation regulating receptors. PAR-1 provides rapid active thrombin binding at low thrombin concentrations, while PAR-4 acts as a slow process at higher thrombin concentrations. Among the purinergic receptors, $P2Y_1$, $P2Y_{12}$ bind to adenosine diphosphate (ADP) and $P2X_1$ to adenosine triphosphate (ATP), thereby enhancing its activation.

Toll-like receptors (TLRs) belong to Pattern recognition receptors (PRRs) and play an important role in immunity and in immune response. During infection, bacteria can also bind directly to platelets through the TLR2 and TLR4 receptors and resulting in upregulation of these receptors. While TLR4 receptor recognizes the lipopolysaccharide (LPS) component of Gram-negative bacteria, TLR2 receptor binds peptidoglycan of Gram-positive bacteria, upon binding the ligand to the receptor, it stimulates platelet adhesion.

CD40L (CD154) belongs to antigen-recognizing receptors and is found in small amounts on the surface of platelets, during platelet activation it comes from the subcellular compartments to the cell surface within minutes and upon activation, surface CD40L is cleaved from the platelet and form the soluble CD40L (soluble CD40L). Both membrane-bound and soluble forms of CD40L may have procoagulant and proinflammatory activity. Soluble CD40L can bind not only to the CD40 receptors but also to the GPIIb/IIIa receptors, thereby inducing platelet aggregation. The Fcγ receptor IIa (FcγRIIa) also belongs to this group, it is a low-affinity receptor of the Fc domain of immunoglobulin G (IgG) and plays a role in immunological protection against bacteria, viruses and parasites. In various autoimmune and alloimmune diseases FcγRIIa clustering induces platelet activation.

The role of platelets in hemostasis and inflammation

The most important function of hemostatic processes is (i) to prevent further blood loss by clot formation in case of vascular wall injury, (ii) keep the blood in a liquid state, and (iii) remove formed clot. Platelets circulate in an inactive state in the bloodstream under normal physiological conditions. During vascular wall damage, extracellular matrix proteins become exposed and this leads to platelet adhesion and activation. The activation process consists of three overlapping phases. In the initial step, platelets interact through their adhesion receptors with a collagen and vWF which were released from damaged endothelial cells, resulting in platelet adherence in a single layer to the wall of the damaged vessel. The α2β1 integrin and GPVI receptors bind directly to collagen, while GPIbα and GPIIb/IIIa receptors bind to collagen through vWF. These processes result in the initiation of signaling pathways and the activation of platelets. During activation, the intracellular calcium level increases, which is accompanied by the release of phosphatidyl serine to the surface these changes facilitate the efficiency of the action of factors and factor complexes (tenase, prothrombinase) and the initiation of coagulation processes. In the second, extension phase, additional platelets are activated and then aggregated this leads secreting, among other things, ADP from the dense granules and P-selectin from the α-granules. Increased expression of P-selectin on the cell surface leads to multiple cell-cell interactions between platelets, white blood cells, and endothelial cells. Secreted ADP and the resulting thromboxane A2 and thrombin activate additional platelets and resulting in increased secretion and activation of the GPIIb/IIIa fibrinogen receptor.

All of this leads to complete platelet aggregation and eventually the formation of a stable blood clot. In the final phase so-called "stabilization phase", platelets reinforce the blood clot and eventually the whole thrombus retracts and occludes the site of vascular injury.

In addition to hemostatic processes, platelets promote chronic inflammatory processes in atherosclerosis, modulate acute inflammatory diseases such as sepsis and other infections and contribute to the exacerbation of autoimmune conditions. The main physiological role of platelets is to preserve endothelial barrier function but they also contribute to the disruption of barrier function in inflammatory processes and thus increase endothelial permeability. Under physiological conditions, platelets do not adhere to endothelial cells. However, in the process of atherosclerosis platelets are activated due to inflammation of the vasculature. In further processes activated platelets adhere to the endothelium and play a role in neutrophil and monocyte adhesion and mediating their endothelial accumulation. As a result of this process, at the site of inflammation the amount of platelet-white blood cell heterotypic aggregates increases. At the same time, activated GPIIb/IIIa expression, α-granule secretion and platelet microparticles formation are increased due to platelet activation through the GPVI immunoreceptor. Platelets as microbial sensors recognize endogenous ligands and microbial pathogen-associated molecular patterns (PAMPs) through their TLR receptors, which also suggests that platelets also respond to damage-associated molecular patterns (DAMPs) that may be released from activated or necrotic cells and may be formed after tissue damage, thus platelets contribute to non-infectious immune diseases. In sepsis, bacterial lipopolysaccharide (LPS) activates platelets through platelet-expressed TLR4 receptors, thereby platelets bind to neutrophil granulocytes and are causing the release of granulocyte nucleus which are leading to the formation of neutrophil extracellular traps (NETs). NETs have not only an antimicrobial effect by capturing and eliminating bacteria and other pathogens. NETs are also able to catalyze and enhance the thrombus formation by activating and aggregating platelets. In addition, increased NET formation is a possible mechanism of platelet-induced vascular damage in sepsis. Both, via the GPVI receptor as well as with LPS-induced activation, platelet-derived microparticles are shedded from the surface of activated platelets and can transport proinflammatory and prothrombotic factors such as CD40L, on this way the signal is transmitting to the target cell thereby, microparticles are participating in the development of thrombosis and transformation of the vasculature. The platelet activating effect of LPS is controversial, which may be due to the fact that the two forms of LPS, S-LPS and Re-LPS are present in varying proportions on the surface of sepsis-causing Gram-negative bacteria and the structure of these LPS forms is different in their O-polysaccharide chain. Heparin-induced

thrombocytopenia (HIT) is an immune response-mediated severe adverse effect of anticoagulant therapy by heparin, such IgG-type autoantibodies are formed that recognize platelet factor 4 (PF4), which binds heparin. Thus formed, heparin/PF4/IgG immune complexes induce platelet activation via FcγIIa receptors, this elicits to enter in large amounts of secreted PF4 in the circulation as a result platelet aggregates are formed. During platelet activation, microparticles (PMPs) are released from platelets, their phospholipid-saturated surface catalyze the formation of thrombin and this process leads to thrombocytopenia and thrombus formation.

Platelet subclasses in hemostasis: aggregating and procoagulant platelets

In addition to adhesion, secretion, and aggregation, platelets also play a role in blood coagulation, controlling thrombin generation, supporting fibrin formation, and regulating clot retraction. During platelet activation platelets have different activation states and correspondingly different surface properties. As a result, different platelet subclasses will perform different tasks. Intracellular calcium levels in procoagulant platelets are elevated, resulting in phosphatidyl (PS) expression and thus increasing the activity of tenase and prothrombinase complexes, thereby regulating thrombin and fibrin formation. During thrombus formation procoagulant platelets are placed on the surface of the thrombus in the process of contraction, which is very critical for spatial control of thrombin and fibrin formation. Similarly to PS expressing platelets, coated-platelets are also formed by strong agonist activation, such as collagen and thrombin. However, coated-platelets are not only characterized by PS expression on their surface but they also binds irreversibly α-granules proteins during activation, thereby increasing their procoagulant activity. In platelets, signaling through the GPVI receptor is the major route of procoagulant platelet formation. On PS-expressed platelets, GPIIb/IIIa is mostly inactive while aggregating platelets show low level of PS and high level of active conformation of GPIIb/IIIa, thereby fibrinogen is bind with high affinity to them, this result in the formation of platelet-platelet or platelet-white blood cell aggregates. Aggregating platelets are involved in the clot retraction during platelet-dependent coagulation.

Receptors that cause the platelet-leukocyte interaction

Cell surface selectins and glycoconjugates mediate the anchoring and rolling process of white blood cells and platelets on the vascular surface. The formation of platelet-white

blood cell heterotypic aggregates is a sensitive marker of platelet activation. Elevated levels of platelet-white blood cell aggregates have been found in acute coronary disease (ACS), sepsis and dialysis. Upon formation of heterotypic aggregates, during platelet activation, P-selectin expressed on the platelet surface and binds with high affinity to glycoprotein ligand-1 (PSGL-1), which is constitutively expressed on the surface of neutrophils, monocytes, and lymphocytes. In the process, P-selectin and PSGL-1 form the major receptor-ligand pair between platelets and white blood cells. This binding allows efficient white blood cell recruitment in case of vascular injury. The ligation of PSGL-1 with P-selectin initiates a signaling cascade in leukocytes that leads to activation of the integrin on leukocytes, macrophage-1 antigen (Mac-1, CD11b, integrin $\alpha M\beta 2$), which binds to platelet GPIbα or indirectly binds to the GPIIb/IIIa receptor through fibrinogen. Activated platelets bind to the PAF receptor on the surface of white blood cells through platelet activating factor (PAF), which also leads to Mac-1 activation. In addition, CD40L on the surface of activated platelets and CD40 on the surface of white blood cells play a role in the formation of aggregates. Platelet-evoked Mac-1 activation leads to the binding and activation of coagulation factor X (FX), so that the platelet-white blood cell binding stimulates the coagulation cascade.

Leukocyte adhesion receptors and their role in various diseases

Adhesion molecules are essential for leukocyte migration, differentiation, initiation of cell-cell interactions and they maintain cell-cell and cell-matrix interactions required for cell motility. In addition to these processes, they can also serve as signaling molecules and activates pathways which are critical for cellular functions. The major families of adhesion molecules involved in leukocyte migration, activation, and differentiation are members of the integrins, selectins and immunoglobulin superfamilies. The interactions of leukocytes and the endothelium are mediated by several families of adhesion molecules, each of those is involved in one phase of the process. The function of integrins is to anchor cells to the extracellular matrix (ECM), to establish interactions during cell-cell adhesion and to direct bidirectional signaling. These properties of integrins make them suitable for regulating complex mechanisms such as cell migration, tissue differentiation, division, inflammation, and coagulation. Most integrins bind to RGD-amino acid sequence (arginine glycine aspartate). This tripeptide occurs not only in ECM macromolecules (collagen, laminin, fibronectin, vitronectin) but is also found in plasma proteins such as fibronectin, fibrinogen, and von Willebrand factor. Central players in the leukocyte adhesion cascade are Mac-1

(αMβ2-integrin; CD11b/CD18) and LFA-1 (αLβ2-integrin; CD11a/CD18) from the β2-integrin family. Mac-1 is a protein present mainly on macrophages, in fact, by complement receptor ligand binding, the macrophage kills by phagocytosis a cell that is considered harmful by the immune system and is labeled (opsonized) with the complement fragment. The main function of LFA-1 is to attach leukocytes rolling along the vessel wall to the endothelial surface. This adhesion allows white blood cells to transmigrate through the vessel wall, thereby developing tissue inflammatory response. Leukocyte integrins are key molecules of the process of immune-mediated and inflammatory such as asthma and atherosclerosis. Another group of adhesion proteins is the immunoglobulin superfamily, which is involved in cell recognition, tethering and intercellular communication. Proteins of the ICAM (intercellular adhesion molecules) group belong to this family, which are mainly expressed on endothelial cells and white blood cells. ICAM-2 binds to Mac-1, LFA-1 and fibrinogen, thereby promoting endothelial transmigration of white blood cells in processes such as extravasation and inflammatory response. In addition to this process, ICAM proteins are involved in many other events of the immune response, e.g. in the process of antigen presentation and initiation of T cell proliferation.

L-selectin (CD62L) is constitutively expressed on white blood cells, it is a receptor for "lymphocyte homing", it plays role in slow down of white blood cells and in cell adhesion, migration and signal transduction. Surface L-selectin is regulated by metalloprotease-dependent shedding of the extracellular domain. Its ligands are glycosylated membrane proteins of the endothelial layer of blood vessels e.g. GlyCAM1 glycoprotein of HEV (high endothelial venules) endothelial cells in lymph nodes. On the other hand, its binding to PSGL-1 is the initial step in white blood cell aggregation and binding to HEV, which initiates transmigration during lymphocyte homing. Selectins play a key role in tumorigenesis and metastasis. This is due to the fact that a large number of selectin ligands are expressed in the vascular endothelium and they are creating a potential entry site for circulating tumor cells. In lymphoproliferative diseases, the level of adhesion proteins is decreased, which contributes impaired transendothelial migration of white blood cells and may be associated with the development of the disease. As a result of white blood activation the adhesion molecules can appear in the plasma and their examination may provide information on the severity of the disease. Elevated levels of soluble L-selectin can be detected during systemic inflammation due to neutrophil activation.

OBJECTIVES

Our purpose was to study the modulation of cell surface receptor expression on platelets and leukocytes after cell activation with inflammatory stimuli and immune mediators.

1. In in vitro experiments we investigate the effect of bacterial endotoxin, LPS and anti-PF4/heparin autoantibody on platelet receptor expression and function.

2. We examine the mechanism of the inhibitory effect of a second generation tyrosine kinase inhibitor upon platelet activation through GPVI

3. We study the regulatory effect of intracellular phosphatase activity on lymphocytes surface receptor expression and function.

MATERIALS AND METHODS

Blood sample collection and sample preparation

Platelet receptor and functional assays

Peripheral blood samples were drawn from healthy volunteers into tubes containing 0.105 M sodium citrate (Becton Dickinson, San Jose, CA). Healthy volunteers were recruited from the staff of the Department of Laboratory Medicine of University of Debrecen and no medication was taken in the two weeks prior to the studies. Informed consent was obtained from all participants in accordance with the local institution review board guidelines. Ethical agreements were provided by the local ethical committee of the University of Debrecen (RKEB/IKEB 4875–2017). Platelet rich plasma (PRP) was prepared from whole blood by centrifugation at 170×g for 10 minutes at room temperature (RT). Platelet poor plasma (PPP) was obtained by centrifugation of the citrated blood sample at 1500×g for 15 min at room temperature (RT). Gel filtrated platelets (GFP) were separated according to previous published protocol.

Leukocytes receptor and function assays

Peripheral blood samples were drawn from patients with chronic lymphocytic leukemia (CLL) and healthy volunteers into tubes containing K_3EDTA (Becton Dickinson, San Jose, CA). Patients were recruited at the Division of Hematology, Department of Internal Medicine at the University of Debrecen. Healthy volunteers were recruited from the staff of the Department of Laboratory Medicine and no medication was taken in the two weeks prior to the studies. Informed consent was obtained from all participants in accordance with the local institution review board guidelines. Ethical agreements were provided by the local ethical committee of the University of Debrecen (RKEB/IKEB 4674–2016). Firstly peripheral blood mononuclear cells (PBMC) were separated on Histopaque-1077 (Sigma-Aldrich, St.Louis, MO) than B-cells were isolated by a magnetic Untouched Human B Cell Separation kit (Life Technologies, Oslo, Norway). Plasma (PPP) was separated from K_2EDTA-anticoagulated blood by centrifugation at 1000g for 15 min at RT.

Effect of LPS on platelet activation

Procoagulant activity assay

Sodium citrated whole blood was incubated with 10 µg/mL S-LPS, Re-LPS (Sigma-Aldrich, St. Louise, MO, USA) or as control with HEPES buffer for 4 hours at 37°C. After incubation PBMC was separated then cells were sonicated. For PCA assay, 100 µL cell lysate was incubated by 100 µL of normal plasma for 1 minute at 37°C then the coagulation was started by adding 100 µL of 25 mM $CaCl_2$. The time to fibrin formation was measured by a KC-1 coagulometer.

Examination of binding of Re-LPS and CD40L expression

Binding of Re-LPS to platelets was checked by flow cytometer. Non activated and TRAP-activated (5 µM) anticoagulated whole blood was stained with FITC-conjugated-Re-LPS (1 µg/mL) and CD42a PerCP for 60 minutes at 37°C in the dark. Samples were fixed by paraformaldehyde (PFA) for 60 minutes after washing with PBS samples were measured by FACScan flow cytometer. Mean fluorescence intensity (MFI) of samples was compared to MFI of unstained- Re-LPS. For CD40L measurements, sodium citrated whole blood was incubated with S-LPS, Re-LPS in 10 µg/mL final concentrations (Sigma, St. Louis, MO, USA) or TRAP (5 µM final concentration) and platelets were stained with CD42a FITC and CD154 PE antibodies and IgG1 PE was used as negative control. Samples were incubated for 60 minutes at 37°C in the dark then were fixed with PFA for 60 minutes after washing with PBS samples were measured by FACScan flow cytometer and analyzed with CellQuest software (Becton Dickinson, Mansfield, MA, USA). In the analysis, platelet marker positive (CD42a +) events were gated.

Platelet aggregation

Platelet number of control PRP was adjusted to 260 G/L by adding PPP. Platelets were stimulated with S-LPS (10 µg/mL), Re-LPS (10 µg/mL) or TRAP (5 µM) and the degree of aggregation was detected by Chrono-Log 700 lumiaggregometer (Chrono-Log Corp. Havertown, PA, USA). In our further studies, we sought an answer to whether S-LPS or Re-LPS is able to potentiate the effect of TRAP. To answer this, co-stimulation was performed during the aggregation test.

Analysis of platelet-derived microparticles (PMP)

Sodium citrated whole blood was incubated with S-LPS or Re-LPS in 10 μg/ml final concentration with or without TRAP (5 μM) for 60 minutes at 37°C then PMP was separated by several successive centrifugations. PPP was first separated from the treated anticoagulated blood by centrifugation at 1500 *g* for 15 min at 20 ° C and then PPP was further spun at 13,000 g for 2 min at 20 ° C to remove platelet membrane fragments. The supernatant was then further centrifuged at 16,000 *g* for 30 min at 20 ° C and the PMPs enriched at the bottom of the tube were washed once with BSCG buffer (129 mM NaCl, 1.6 mM KH 2 PO 4, 14 mM Na citrate, 11 mM glucose, 10 mM NaH 2 PO 4 pH: 7.30). This was followed by another centrifugation at 16,000 *g* for 30 minutes at 20 ° C. After centrifugation, 90 % of the volume of the supernatant was removed and the number of PMPs was determined from the remaining 10 % after labeling. PMP samples were stained with annexin V FITC and CD41a PeCy5 and measured by FACScan flow cytometer. PMPs were collected for 30 seconds at low flow rate and CD41a and annexin V positive events were analyzed and the absolute number was reported in PMP / μL plasma.

Platelet activating potential of HIT antibodies

Functional HIT test

Two types of laboratory approaches are used for the diagnosis of HIT: (i) immunoassays for the detection of antibodies against PF4–heparin complex, and (ii) functional assays to detect the platelet activating potential of HIT antibodies. Among the functional assays, flow cytometric test examines the ability of HIT antibodies to activate control platelets in the presence of therapeutic concentration of heparin. PS expression and PMP formation were determined. In the test, 10 μL of patient plasma was added to 10 μL of control PRP. The mixture was incubated for 30 minutes at 27 °C in the presence of therapeutic (0.3 IU/mL) and excessive dose (100 IU/mL) of UFH in a final volume of 50 μL adjusted by PBS. After a 30-min incubation at 27°C, 5μL aliquots were removed from each tube and incubated in the presence of 5 μL phycoerythrin (PE)-labeled 20-fold diluted anti-CD41 antibody (Dako, Glostrup Denmark) for platelet identification and 1 μL annexin V-FITC (Becton Dickinson, Mansfield, MA, USA) for the detection of activated platelets. The samples were adjusted to a final volume of 50 μL with 0.02 M HEPES buffer (pH 7.3) containing 2.2 mM $CaCl_2$. After 15 minutes incubation at room temperature in the dark,

samples were supplemented with 400 μL $CaCl_2$-containing HEPES buffer (pH 7.3) and analyzed by FACScan flow cytometer (Becton Dickinson, Mansfield, MA, USA); platelet acquisition time was 1 min with high flowrate. Platelets were identified by the immunofluorescence of the PE-labeled anti-CD41 antibody. In negative control samples, PRP without patient plasma but with 0.3 IU/mL UFH, <5% of platelets demonstrated annexin V-binding. The result of the annexin V-binding assay was considered positive if: (i) at least 11% of platelets were annexin V positive after treatment with 0.3 IU/mL heparin, (ii) and the ratio of the percentage of annexin V positive platelets generated in the presence of 0.3 IU/mL and that of 100IU/mL of heparin was more than 1.5. PMPs produced by activated platelets were identified by size-selection based on their forward (FSC) and side scatter (SSC) properties. The result of the PMP assay was expressed as the ratio between the number of PMPs generated at 0.3 IU/mL and 100 IU/mL of heparin. Posi-tive result was considered when the PMP ratio was 1.5 or higher.

Effect of dasatinib on platelet activation through GPVI receptor

Collagen- induced platelet aggregation and measurement of ATP release

Citrated whole blood from healthy volunteers was pretreated with dasatinib or nilotinib then for the assessment of platelet functions PFA-100 (Siemens, Deerfield, IL, USA) CT measurements were carried out with CEPI and collagen/ADP (CADP) cartridges. For platelet aggregation test PRP was pretreated with different concentration of dasatinib (0, 5, 10, 50, 100, 150, 200 és 400 nM) for 10 minutes at at 37 °C. Platelet aggregation and secretion induced by 1µg/mL fibrillar collagen (Takeda, Linz, Austria) were tested in a Chrono-Log 700 lumi-aggregometer (Chrono-Log Corp., Havertown, PA, USA). Platelet count in platelet-rich plasma (PRP) was set to 260 ×109/L by platelet-poor plasma (PPP). The aggregation process was followed for 8 min and results were expressed as percen-tage of maximal change in light transmission (ΔTmax%). ATP release during platelet activation was determined by bioluminescence method using luciferin-luciferase reagent (BiothermaAB, Handen, Sweden). Maximal ATP secretion was expressedasµmol ATP/1011platelets.

Measurement of PS expression

PRPs from healthy volunteers were pretreated with dasatinib (10 and 100 nM) or nilotinib (5000 nM) and after that platelets was activated by convulxin (12.5 ng/mL) for 15 minutes at 37 °C. Platelet PS expression was determined by annexin V binding to platelet

surface using FITC-conjugated annexin V and for platelet identification PE-conjugated CD41 monoclonal antibody was used. Five microliter of PRP was stained with 5µL of annexin V-FITC and 5µL of CD41-PE in 35µL annexin V binding buffer (it provides calcium for binding of annexin V), and the mixture was incubated for 15 minutes at RT in the dark. After the incubation time, 500 µl of annexin binding buffer was added and samples were measured on an FC500 flow cytometer and analyzed with Kaluza software (Beckman Coulter, CA, USA). Platelets in the CD41 positive gate were analyzed and the amount of annexin V positive platelets was given in percent.

Coated-platelet determination

Gel filtered control platelets were pretreated with dasatinib (0, 5, 10, 50, 100, 150, 200 és 400 nM) or nilotinib (0, 125, 250, 1000, 2500 és 5000 nM) for 10 minutes at 37 °C. Subsequent platelet activation was assessed in a total volume of 100 µl, containing a Mix buffer (79µl), GFP with a concentration of 40 g/L (10µl), 1µl of biotinylated-fibrinogen (100µg/ml) (Sigma-Aldrich), and 10µl agonists mix with final con-centration of convulxin at 125 ng/ml (Pentapharm, Basel,Switzerland) and 0.5 U/ml thrombin (bovine thrombin;Sigma-Aldrich). The Mix buffer was prepared with the following components: 10 mM of HEPES, 1 mg/ml of BSA, 2.5 mMof $CaCl_2$, 1.25mM of $MgCl_2$, and 150 mM of NaCl. Reaction was assessed for 10 min at 37°C, then was stopped with 200µl of 1% (wt/vol) paraformaldehyde and fixed for 20 min at RT. After fixation, 3.5 ml of PBS containing 1 mg/ml of BSA (PBS/BSA) was added, and the sample was centrifuged at 1500 ×g for 15 minutes. The pellet was resuspended in 200 µl PBS/BSA labeled with antiCD41a-PECy and Streptavidin-PE for 20 min at RT in dark and after washing with PBS, it was centrifuged as mentioned above and finally resuspended in PBS. The levels of coated-platelets were determined in percentage (%) of platelet population in the CD41a positive gate. All flow cytometric analyses were performed on a BD FACS-Canto II instrument (Becton Dickinson, Mansfield,MA, USA) and data were analyzed via BD FACSDiva software (6.1.3 version).

Thrombin generation test

PRPs from healthy volunteers were pretreated with dasatinib (10 and 100 nM) or nilotinib (5000 nM) and after that platelets was activated by convulxin (12.5 ng/mL) for 15 minutes at 37 °C. Thrombin generation was measured in PRP using Fluoroskan Ascent FL fluorimeter with Thrombinoscope reagents and software (Thrombinoscope BV, Maastricht, The Netherlands). Assays were carried out according to the manufacturer's instructions. Into

wells of a black plate, 80µL of pretreated PRP and 20µL of standard preparation containing1 pM recombinant tissue factor (PRP reagent)/Thrombin calibrator were pipetted and after incubation for 10 min at 37◦C, the thrombin generation was started by adding 20µL of FluCa (fluorogenic substrate and calcium in buffer). Fluorescence was detected and the thrombin generation curve was generated. The kinetics of thrombin generation was characterized by lagtime, time to peak, while the quantity of generated thrombin was described by thrombin peak end endogenous thrombin potential (ETP).

Detection of activated conformation of GPIIb/IIIa by PAC 1 binding

PRPs from healthy volunteers were pretreated with dasatinib (10 and 100 nM) or nilotinib (5000 nM) and after that platelets was activated by convulxin (12.5 ng/mL) for 15 minutes at 37 °C. Active conformation of the integrin αIIbβ3 was determined by binding of FITC-conjugated PAC1 and PECy5-conjugated CD41a antibodies. Measurement of active conformation of the integrin αIIbβ3 was performed on FC500 flow cytometer and analyzed with CXP software (Beckman Coulter, CA, USA).

Investigation of Clot Retraction

In the in vitro experiments, 900µL of PRP was preincubated with 100µL of buffer control or different concentrations of dasatinib or nilotinib for 10 min at 37◦C in a water bath and subsequently was activated by convulxin for 15 min at 37◦C. In a glass tube, 1000 µL of TKI-pretreated and activated PRPs from an in vitro experiment, and similarly to this, 1000µL PRPs from dasatinib/nilotinib treated CML patients were incubated with 100µL of 250 mM CaCl2 (at a final concentration of 22.7 mM) for 60 min at 37°C in a water bath. At the end of incubation, photos were taken to document clot formation and the volume of the extruded serum was determined.

Examination of SFKs by western blot

Gel filtered control platelets were pretreated with dasatinib (0, 10, 100 nM) and these TKI pretreated platelets were activated by convulxin (12.5 ng/mL). Gel filtrated platelets (4×10^7 from each sample) were lysed with lysis buffer containing 1% TritonX-100 in PBS supplemented with a cocktail of tyrosine phosphatase inhibitor from Sigma (St. Louis, MO, USA). Platelet lysates were separated by polyacrylamide gel electrophoresis and subjected to western blotting. Specific phosphorylation of Lyn, Fyn, and Src kinases were visualized using

phosphospecific antibodies (Ab) and biotinylated secondary Ab followed by avidin-biotin complex for 30 min. Bands were demonstrated by enhanced chemiluminescence (ECL).

Investigation of receptors, function and mechanism of leukocytes

Determination of cell surface L-selectin and TACE/ADAM17 expression

For the analysis of cell surface L-selectin and TACE expression of normal B-cells and CLL cells, we used afour-color staining. CD19-PC7 and CD5-PerCPCy5.5 antibodies were used for cell identification. We used FITC-labeled TACE and PE-conjugated CD62L monoclonal antibodies for detection of cell surface expression of TACE and L-selectin. Mouse IgG2a-FITC was used as an isotype control for the investigation of TACE expression. A quantitative flow cytometric determination of cell surface L-selectin was carried out by a direct immuno fluorescence assay using Quantibrite™ PE fluorescence quantitation kit. Results were expressed as antibody binding capacity (ABC). Cells were labeled for 30 minutes at RT in the dark. Then, they were washed twice with PBS and finally were fixed in 500 µL 1% PFA.

Determination of phosphorylated p38MAPK

Pp38MAPK expression was detected by flow cytometry according to the following procedure. PBMCs from controls and patients with CLL were stained with cell surface markers and were immediately fixed with BD Phosflow™ Fix buffer I. in a 37°C waterbath for 10 min. Thereafter, cells were washed with BDPhosflow™ Perm/Wash buffer I. and resuspended cells were stained with Mouse Anti-pp38 MAPK (pT180/pY182)-PE antibody for 30 minutes at RT in the dark. After washing cells were resuspended in BD Pharmingen™ Stain buffer and measured by FC500.

Measurement of L-selectin from plasma by ELISA

Plasma from patients with CLL and healthy volunteers were separated from K2EDTA-anticoagulated blood by centrifugation at 1,000 g for 15 minutes at RT. Soluble L-selectin was measured from plasma samples using commercially available enzyme-linked immunoassay according to the manufacturer's instructions.

Western blot analysis of soluble L-selectin

Briefly, plasma of CLL patients was purified from immunglobulins by Protein G Sepharose C. After centrifugation anti-human L-selectin antibody and another aliquot of Protein G Sepharose were added to the supernatant of the immunglobulin free plasma. Immuno-precipitation was done overnight at 4°C. After washing, precipitates mixed with Loading Dye (61.90 mM TRIS, 2%SDS, 10% glycerol, 0.025% bromphenol blue) were boiled for 5 min. Samples were applied to SDS-PAGE using 10% polyacrylamide gel. Proteins were blotted to nitrocellulose membrane that was blocked in 3% gelatin. Subsequently the membrane was incubated with biotinylated anti-human L-selectin antibody for 90 minutes followed by avidin-biotin complex for 60 minutes. Bands were visualized by enhanced chemiluminescence (ECL).

Protein phosphatase assay

Magnetically separated normal B-cells and CLL-Bcells (5×105) were suspended in lysis buffer containing 50 mM Tris–HCL (pH 7.4), 150 mM NaCl, 1% TritonX-100 and 0.5% protease inhibitor cocktail. The lysate was sonicated, and then centrifuged at 15,000 g at 4°C for 10 min. The phosphatase activity of the supernatants was determined at 30°C using 1µM 32P-labeled 20 kDa light chain (32P-MLC20) of turkey gizzard myosin as substrate in the absence or presence of PP1inhibitor-2 (I-2, 2µM). The reaction was initiated by addition of the substrate and after 10 min incubation was terminated by the addition of 10% TCA and 6 mg/ml BSA. Following centrifugation, the released ^{32}Pi was determined from the supernatant in a scintillation counter (Perkin Elmer). The total phosphatase activity in normal B-cells was regarded as 100%.

In vitro experiment with CLA and p38MAPK inhibitor

These experiments were carried out in suspension of magnetically separated normal B-cells and B-CLL cells. Cells were incubated with DMSO or CLA in different concentrations at 37°C for 30 minutes. For those experiments, where we wanted to inhibit p38MAPK, cells were preincubated with 5µM SB203580—as specific inhibitor of p38MAPK—before CLA treatment. After treatments pp38MAPK level and surface L-selectin were examined as describe above.

Statistical analysis

The statistical analysis was performed with GraphPad Prism 4.0 version. Normality of the data was evaluated by the Kolmogorov-Smirnov tests. Data were expressed as mean ± SD or mean ± SEM. Predetermined pair-wise differences were analysed by paired t test and incase of a non-normal distribution, the Wilcoxon matched paired test was used. For non pair-wise comparison, unpaired t test (Gaussian distribution) and Mann-Whitney test (non Gaussian) were used. ANOVA (Gaussian distribution) or Kruskal-Wallis test (non Gaussian distribution) were used for multiple group comparison. P values less than 0.05 were considered as statistically significant.

RESULTS

In inflammatory processes, endothelial cells synthesize inflammatory cytokines, such as platelet activating factor (PAF) induced by thrombin or other inflammatory stimuli. In addition, all cells that are involved in protecting the body, such as platelets, neutrophils, monocytes and macrophages, are also capable of synthesizing PAF. In the inflammatory response, PAF mainly regulates the cell-cell interaction, on the one hand activates polymorphonuclear (PMN) cells and induces platelet activation and aggregation.

Platelet activation can occur via two main signaling pathways. During a "classic" pathway the activation is elicited by collagen, thrombin, ADP, adrenaline, or thromboxane A2. In the other signaling process inflammatory mediators such as PAF or LPS cause platelet activation. During activation, platelets undergo several morphological and biochemical changes. These changes allow platelets to perform their biological function, which can be well traced by various methods.

In our previous studies, we showed platelet P-selectin and CD40L expression were increased and platelet-derived microparticle formation (PMP) is enhanced during activation by PAF in concentration dependent manner (unpublished data).

Effect of LPS on platelet activation

We investigated the effect of LPS, as an inflammatory stimulus, on platelet activation and function. First, to evaluate the biological activity of these LPS forms, we tested their procoagulant properties in a recalcified, LPS-stimulated mononuclear cell suspension. Re-LPS and S-LPS (10 µg/mL) significantly ($P<0.05$) decreased the mean clotting time compared with the non-activated control. Furthermore, we have shown that Re- LPS interacted directly with platelets as FITC-labeled Re-LPS binding resulted in significantly increased FL-1 mean fluorescence intensity (MFI) of the positive cells (MFI, 49.7±8.3; $p=0.01$) compared with control platelets with unlabeled Re-LPS (10.2±2.4), an even larger increase in MFI was observed with TRAP (10 µM) (data not shown). Next, we examined whether Re-LPS and S-LPS affected platelet aggregation. Neither LPS form alone (0.1–10 µg/mL) induced platelet aggregation. In contrast, 1µg/mL of Re-LPS but not S-LPS augmented submaximal TRAP-induced (5µM) platelet aggregation. The expression of CD62P and CD40L as sensitive activation markers was also analyzed after activation with up to 10 µg/mL of Re-LPS and S-LPS in parallel. No increase in the level of P-selectin-positive platelets was induced by either LPS form compared with the untreated sample (9.0±4.0%,

9.5±5.0% vs. 7.6±3.0%; data not shown). On the other hand, a statistically significant elevation in CD40L expression (2.7±1.9%; $p<0.05$) with high MFI values (172.5±80.0; $p<0.001$) was detected but only at 10 g/mL of Re-LPS vs. the negative control. In addition, TRAP also caused a substantial increase in CD40L expression (33.6±4.9%; MFI=33.6±4.7). Interestingly, S-LPS did not raise the level of surface CD40L. We found a significant increase ($P<0.05$) in platelet-derived microparticle (PMP) levels induced by Re-LPS (10 μg/mL) (450±170 PMPs/μL plasma); however, S-LPS did not alter the PMP number (273±144 PMPs /μL plasma vs. 227±95 PMPs /μL plasma in unstimulated sample). These levels were below those we observed in TRAP-treated (5μM) samples. We also wondered if Re-LPS at even lower concentrations could potentiate PMP generation in the presence of TRAP by synergism. PMP levels were significantly enhanced already at 1 μg/mL of Re-LPS during co-activation (1384±299 PMPs/μL plasma; $p<0.05$ compared with the sample with TRAP alone). Of note, S-LPS had neither synergistic nor inhibitory effects on the PMP level under the same conditions (983±120 PMPs/μL plasma).

Effect of HIT antibody on platelet activation and function

The functional assays were performed in samples of all ELISA (anti-PF4/heparinIgG) positive patients (OD>0.5; n=40) in addition to 40 randomly chosen ELISA negative patients (OD<0.3), 11 borderline/gray zone (OD=0.3-0.5) cases. Similarly to controls, patients with borderline or negative ELISA results had negative functional assay results confirming HIT negativity. Out of the 40 ELISA positive patients, the Annexin V-binding assay was positive in 17, while the PMP assay was positive in 14 cases. HIT negative control plasma did not activate donor platelets, however, plasma sample of a HIT patient stimulated donor platelets in the presence of pharmacological heparin concentration (0.3 IU/mL). The fact that in the presence of excessive amount of heparin (100 IU/mL) platelets were not activated—or significantly less activated—proves the strict heparin concentration dependency of platelet activation, the reason of this high concentration of heparin prevents the formation of an immune complex that would activate platelets.

Furthermore, the association between the IgG isotype ELISA OD values with the outcome of the annexinV-binding assay and with PMP assay was analyzed. There was a positive association between the ELISA OD values and the functional assay positivity (annexin V binding: $p=0.0001$; PMP: $p=0.0002$). It means that the higher the IgG isotype ELISA OD value was measured, the higher the probability of a positive functional assay result was found. Since the most severe adverse event associated with HIT is thrombosis, we

statistically analyzed the relationship between heparin-dependent antibody levels and the frequency of thrombotic events. The higher level of heparin-dependent antibody was associated with increased probability of thrombosis.

Effect of dasatinib on platelet activation and function

Normal platelets of healthy volunteers were incubated with different dasatinib and nilotinib concentrations, and coated-platelet formation, PFA-100 CT with CEPI and CADP cartridge, and collagen-induced platelet aggregation and ATP secretion were studied. Incubating normal GFP with dasatinib resulted in a significant reduction in coated-platelet formation even at the low therapeutic range, i.e., 50 nM ($p \leq 0.01$), and a dose-dependent further decrease was observed at higher dasatinib concentrations.

Dasatinib at 400 nM elicited prolongation of PFA CT with CEPI cartridge but not with CADP cartridge.

Dasatinib concentrations up to 100 nM did not inhibit platelet aggregation by collagen, but 150 nM dasatinib pretreatment significantly prevented ($p<0.05$) platelet aggregation and also prevented ATP release. Nilotinib was used as control and resulted in no change of coated-platelet formation, PFA-100 measurement and platelet aggregation.

We intended to investigate whether dasatinib also affects the platelet procoagulant activity and thereby coagulation. For this reason, for pretreatment dasatinib was used at 10 and 100 nM while nilotinib was used at 5000 nM final concentration for 10 minutes at 37°C. One part of pretreated platelets were activated by GPVI agonist, convulxin at 12.5 ng/mL final concentration for 15 min at 37°C and other part of platelets was left non activated (resting platelets). After treatments, PS expression, platelet-dependent thrombin generation and clot retraction activity of platelets were examined. Pretreatment of platelets with 100 nM dasatinib caused significant reduction of PS expression even in non-activated samples. The GPVI agonist convulxin caused a definitely higher PS expression compared to non-activated platelets, which was abolished by 100 nM dasatinib pretreatment. In the same experiments, the modulatory effect of dasatinib on the thrombin generation was investigated. The thrombin generation test (TGT) can be executed in PRP, in this case the activator is only TF and the PL is provided by the activated platelet surface lipids. From the above mentioned samples we investigated the kinetic of thrombin generation: (i) Lag time, the moment at which thrombin generation starts (minutes); (ii) Time to Peak, the time until the Peak (minutes) and quantity of generated thrombin: (iii) Peak Thrombin, the highest thrombin concentration (nM); (iv) Endogenous Thrombin Potential (ETP), the area under the curve (nM×min). Thrombin

formation was faster, and the lag time and time to peak values were significantly shorter in convulxin activated samples compared to the non activated samples. Activation by convulxin increased the peak thrombin but did not affect the ETP values. High concentration of dasatinib (100 nM) significantly delayed thrombin generation in the convulxin activated samples but did not affect the non activated samples. In addition, 100 nM dasatinib also significantly attenuated the peak thrombin but had no effect on the ETP in the convulxin activated sample. Low concentration of dasatinib (10 nM) and nilotinib did not affect the parameters of TGT.

From the same samples we investigated the level of activated GPIIb/IIIa by PAC 1 binding using flow cytometer. Dasatinib at 100 nM significantly reduced the level of activated integrin in non activated sample and strongly inhibited the integrin activation that was elicited by the GPVI receptor agonist, convulxin. Clot retraction was assayed for 60 min. At the end of the incubation period, the non activated sample displayed intense clot retraction. In samples, where platelets were activated by convulxin, the clot contraction was less intense. However, 100 nM dasatinib pretreatment significantly increased the volume of the extruded serum in the convulxin activated sample. Nilotinib and 10 nM dasatinib pretreatment had no effect on integrin activation or clot retraction.

The decreased platelet response to the GPVI agonist convulxin suggested that dasatinib may influence platelet signaling via the inhibition of SFKs. In this reason control GFP was treated by dasatinib at 0, 10, and 100 nM final concentrations, and these TKI pretreated platelets were activated by convulxin. In case platelets were not pretreated with dasatinib but were activated by convulxin for 15 minutes, a decreased phosphorylation level was observed in both the C-terminal tail (inactive form) and the activation loop (maximally active form) of SFKs. In line with the previous data, convulxin could result in an attenuated effect in the presence of 10 nM dasatinib but 100 nM dasatinib abolished this effect

Modulatory effect of phosphatase activity on L-selectin expression

In case of CLL patients, surface L-selectin analysis was conducted on $CD5^{+}/CD19^{+}$ B-CLL cells while in control samples L-selectin expression was investigated on $CD5^{-}/CD19^{+}$normal B-cells.

We have found that surface L-selectin was significantly reduced on B-CLL lymphocytes in Rai 0 stage of CLL, compared to normal B-cells. Simultaneously soluble L-selectin was significantly elevated in the plasma of B-CLL patients but when the soluble L-selectin level of plasma of CLL patients was normalized for 10^6 leukocyte counts, soluble L-

selectin values became lower in CLL patients. In cases of patients, where absolute lymphocyte count was above 32 G/L, B-CLL cells expressed low surface L-selectin levels and at the same time plasma samples derived from these patients displayed elevated soluble L-selectin values.

L-selectin expression on T-cells in the CLL group was similarly lower but the differences were less pronounced. The soluble form of L-selectin can derive from both lymphocytes and neutrophils as could be detected in samples containing considerable number of neutrophils. Nevertheless, since the mean neutrophil count in the investigated samples was 4.5 G/L, this represented a neglectable quantity compared to the mean absolute lymphocyte count (43.6 G/L). By flow cytometry 78% of these lymphocytes were B-CLL cells, thus soluble L-selectin represented primarily the B-CLL cell derived protein.

Next, we performed studies to help understand the mechanism of decreased L-selectin expression. Our results showed that TACE expression was significantly lower on the surface of CD5+/CD19+ malignant B-cells while pp38MAPK level was significantly higher in these cells compared to normal B-cells.

After that, phosphatase activity of B-CLL and normal B-cell lysates was assayed in the absence and presence of PP1 inhibitor-2 (I2), accordingly total and PP2A phosphatase activities (the activity in the presence of I2) were determined.

Subsequently, in in vitro experiments, normal B-cells and malignant B-cells from CLL patients were pretreated with a calyculin A (CLA) phosphatase inhibitor, and then surface L-selectin expression and pp38MAPK levels were examined. Leukemic B-cells from CLL patients showed higher basic pp38MAPK level compared to normal B-cells, after CLA treatment the pp38MAPK level was elevated in a dose-dependent manner in both groups but the pp38MAPK level was higher in B-CLL cells compared to normal B-cells at all investigated CLA concentrations. Similarly, a rapid downregulation of L-selectin was observed by CLA treatment with different kinetics in the two groups. In case of malignant B-cells, L-selectin almost entirely was shed from the cell surface at 10 nM CLA concentration.

In the following studies, normal B-cells were preincubated with a highly specific inhibitor of p38MAPK (SB203580) at 5μM concentration before CLA treatment. The p38MAPK inhibited cells showed decreased level of pp38MAPK after CLA treatment and 5μM SB203580 almost completely inhibited the CLA induced phosphorylation of p38MAPK at 5 nM CLA concentration (data not shown). Similarly to this change the CLA-induced shedding of L-selectin was also attenuated at 5 nM CLA by p38MAPK inhibitor.

DISCUSSION

Platelets are known to have a complex function, major regulators of cellular hemostatic processes, contribute to hemostatic plug formation and accelerate the coagulation system. During these processes, platelets come into contact with various cell types, monocytes, neutrophils, endothelial cells and smooth muscle cells and contribute to the pathogenesis of atherosclerosis, vascular inflammation and other inflammation-mediated diseases such as sepsis. In addition to these diseases, during infection platelets come into contact with microorganisms and neutrophils. As a result NETs are formed, which on the one hand provide protection against the invasion of pathogens and on the other hand participate in the development of infection-mediated thrombosis.

Technically and methodologically well-developed laboratory functional tests are required to characterize platelets and we can easier understand their complex role in pathological processes. Platelet activation can result in "classical" activation, which means platelet aggregation, clot retraction and increased activation markers including P-selectin, CD40L and activated GPIIb / IIIa expression. The "alternative" biomarkers include PMPs, heterotypic aggregates, PS expression and functional assays such as HIT assay, coated-platelet formation and platelet-dependent thrombin formation.

In addition to inflammatory and immune mediators, we investigated the effect of TKIs on platelet receptors, signaling and function and we studied the modulation of L-selectin in CLL on leukocytes.

In our in vitro study, we examined the effect of two forms of LPS, Re-LPS (Salmonella minnesota, Re595) and S-LPS (Escherichia coli, 0111: B4) on platelet activation and function. Our experiments with LPS were done as previously contradictory results were published. In our studies we found that Re-LPS and S-LPS at a concentration of 1 µg/mL alone did not induce classical platelet activation, but Re-LPS was able to potentiate the platelet aggregating and PMP-generating effect of submaximal concentrations of TRAP and only very high concentration (10 µg/mL) induces an increase in CD40L expression and PMP formation. Our results are line in with a previous study showing that LPS forms with different structures from different bacterial strains below 10 µg/mL concentration alone could not induce classical platelet activation, however, LPS at very high concentrations (100 µg/mL) enhances P-selectin expression. Contrary to our results, Vallance et al. found, simultaneous stimulation of platelets with a supraphysiological concentration of S.minnesota LPS (2

μg/mL) and with a GPVI receptor agonist, CRP-XL (collagen related peptide-XL), resulted in decreased P-selectin expression.

In summary, we have shown that platelets respond differently to the two forms of LPS, possibly due to the different structure of the O-antigen region of LPS. On the other hand, in addition to structural differences, the relationship between TLR4 and soluble plasma proteins such as CD14 and LBP (lipopolysaccharide binding protein) is also need.

In our next study, we examined the effect of IgG-type HIT antibodies on platelet activation. A side effect of heparin therapy may be to induce an immune response that produces IgG-type antibodies in the bloodstream that bind to the heparin-PF4 complex. The formed complex of heparin, PF4 and antibody binds to the FcγRIIa of platelet through Fc-part of the antibody and activates platelets; this leads to a hypercoagulable state with the development of thrombocytophenia, which might result in thrombosis. In our in vitro study, we examined the effect of HIT antibodies- present in the plasma of HIT suspected patients- on the procoagulant potential of control platelets and the formation of PMPs in the presence of therapeutic concentration heparin using a functional test. Then the results of the flow cytometric functional test were compared with the OD values of HIT antibodies which were determined by ELISA. Similarly to Warkentin et al., we found a significant positive correlation between ELISA OD and the positivity of the functional test in case of both examined activation markers. In addition, we showed a high antibody titer (expressed in OD) was significantly positively correlated with the possibility of thrombosis. This finding was consistent with data reported in the literature as 69% of the ELISA and SRA (Serotonin Release Assay) positive patients suffered from thrombosis. In our study 71% of patients with ELISA and annexin V-binding functional test positivity had thrombosis.

In summary, our results suggest that the functional flow cytometric HIT test, which detects annexin V binding and PMP formation, can reliably predict the clinical outcome of the side effect of heparin treatment.

Since the introduction of BCR-ABL tyrosine kinase inhibitors, the overall survival of patients with chronic myeloid leukemia has markedly improved. However, long term use of these drugs results in various adverse events. Among second generation TKIs, dasatinib treatment is often complicated by hemorrhagic events, the most common being gastrointestinal bleeding, while nilotinib activates platelets and endothelium on this way potentiates platelet adhesion and thrombus formation.

In our in vitro study, we examined the effect of clinically relevant concentrations of dasatinib and nilotinib on platelet activation and function. In line with the results of previous

in vitro studies, we found that dasatinib dose-dependently attenuated platelet activation and therapeutic plasma concentrations (150 nM) of dasatinib significantly inhibited collagen-induced platelet aggregation and ATP secretion and only at very high concentration (400 nM) prolonged the PFA-100 occlusion time with the collagen/adrenaline cartridge. In addition to these platelet functional tests, which provide information on the role of platelets in primary hemostasis, we also examined the formation of coated-platelets, a functional test is not generally used in platelet diagnostics. In our studies, we found that 50 nM dasatinib pretreatment resulted in significantly less coated-platelet formation, based on this we concluded coated-platelet assay is more sensitive than lumi-aggregometry to investigate the inhibitory effect of dasatinib to GPVI receptor-induced platelet activation.

Platelets play a central role in the primary hemostasis and are also actively involved in cell-based thrombin generation. In this regard, we sought to answer whether dasatinib affects the formation and function of PS-expressing procoagulant platelets. To answer this question, no data have been reported in the literature so far, so in our next in vitro study we examined the effect of clinically relevant concentrations of TKI on procoagulant platelet formation. Our experimental results clearly showed that the higher end of the therapeutic range (Cmax), 100 nM dasatinib pretreatment significantly reduced PS expression in non activated samle, but the inhibitory effect was more pronounced in the convulxin-induced activation response in all tests, PS expression, thrombin formation, integrin activation and clot retraction. In contrast, therapeutic concentration of nilotinib did not affect platelet function in any of the tests. These results were confirmed by the results of a publication in 2020, in which the authors found that dasatinib inhibits procoagulant platelet formation and mitochondrial membrane depolarization as well as collagen receptor-induced GPIIb/IIIa activation.

BCR-ABL tyrosine kinase inhibitor, dasatinib may inhibit SFK kinases Lyn, Fyn, and Src, which are regulators of platelet signaling and activation through the GPVI receptor. However, it cannot be concluded from these data that dasatinib exerts its inhibitory effect on platelet function through inhibition of SFKs. To clarify this issue, we examined the phosphorylation status of SFKs in dasatinib pretreated and convulxin activated platelets by Western blot. In line with previous reports, dasatinib was found to inhibit the inactive and active forms of all three kinases even at very low concentrations of dasatinib (10 nM) in non activated sample and high therapeutic concentration of dasatinib (100 nM) completely inhibited SFKs. These effects were the same as we observed for PS expression, when platelets were pretreated with 10 nM dasatinib, convulxin was able to exert its activating effect on platelets. However, 100 nM dasatinib completely abolished this effect.

It has long been observed that dasatinib treatment may lead to bleeding symptoms in CML, which may be due to various reasons. Dasatinib treatment may be associated with mild thrombocytopenia and as can be seen from our results, dasatinib may cause platelet dysfunction but no association was found between bleeding symptoms and the impaired platelet function and it was concluded that the occurrence of bleeding cannot be predicted by in vitro platelet aggregation tests. In addition, dasatinib may exert an effect on other elements in the circulation. Dasatinib triggers a transient increase in vascular leakage and causes eryptosis which is associated with an increase of PS expression. The effect of dasatinib on circulation is complex, and the clinical outcome of bleeding might depend on which effect is more pronounced, e.g, inhibition or promotion of PS expression on different types of cells.

In summary, our work demonstrated the off-target effects of dasatinib on platelet function and explored a mechanism that could lead to the observed phenomenon. Dasatinib pretreatment inhibited platelet aggregation and procoagulant activity of plateles during the GPVI receptor activation. These effects may contribute to the hemorrhagic consequences of dasatinib-treated patients through endothelial abnormalities or damage when the GPVI agonist subendothelial collagen is released.

In case of vascular injury and independently that, in many inflammatory processes or malignancies one of the excellent markers of thrombotic diseases is the formation of platelet-leukocyte heterotypic aggregates. All types of leukocyte express an adhesion receptor. L-selectin is expressed by all types of leukocyte and its structure is very similar to the structure of platelet P-selectin but L-selectin is a constitutively expressed protein.

Leukocytes interact with platelets during thrombotic processes so in our next study, we examined L-selectin expression on leukocytes in normal and pathological samples. We studied L-selectin expression in the most common adult leukemia which is chronic lymphocytic leukemia. Our hypothesis was that in this disease, which has a homogeneous morphological appearance and variable clinical stages and prognosis, the adhesion receptors may be associated with the clinical stages of the disease.

In numerous hematological malignancies, unrestricted activation of oncogenic kinases occur that can either be achieved by the constitutive activation of further kinases or via the inactivation of their antioncogenic phosphatases. Elevated level of SET-oncoprotein has long been known in chronic lymphocytic leukemia, it causes the development of malignancies mainly through the inhibition of the antioncogenic protein phosphatase 2A, in this process Lyn tyrosine kinase enhances SET-mediated inhibition of PP2A. In CLL, the progression of the disease is mediated by on leukemic cells expressed adhesion receptors.

The primary aim of this work was to delineate mechanisms that determine surface expression of L-selectin in CLL. Similarly to previous studies, we found that L-selectin expression on the surface of malignant B-cells was decreased in ex vivo patient samples and it was associated with elevated level of soluble L-selectin. Surface L-selectin expression correlated with the clinical status of the CLL patient as determined by the Rai-stage. Thus, a lower level of surface L-selectin expression corresponds to a more severe Rai-stage. This observation was in line with previous results where low cell surface L-selectin expression on malignant CLL cells was found to have negative prognostic value. Prognostic markers used in CLL include lymphocyte doubling time, CD38 and ZAP70 expression, IGHV status and certain cytogenetic abnormalities. Shanafelt et al. found the expression of CD49d adhesion protein in CLL as an independent predictor of overall survival (OS) and also a better prognostic factor than ZAP-70, IgVH status, or cytogenetic abnormalities. The proliferating capacity of CD49+ CLL cells is higher than CD49- cells, so the knowledge of the pattern of CD49 may improve the prognostic significance of this biomarker. Sopper et al. detected decreased L-selectin expression on T-cells as well as elevated plasma L-selectin levels in another group of leukemia patients this phenomenon reduced the efficacy of tyrosine kinase inhibitor therapy in CML. In our study we detected decreased cell surface L-selectin, decreased phosphatase activity (total and PP2A), increased phosphorylated p38MAPK levels and decreased cell surface TACE (Tumor necrosis factor alpha, responsible for surface L-selectin cleavage) in CLL B-cells. In our in vitro experiments, the phosphatase inhibitor CLA induced phosphorylation of p38MAPK and downregulation of cell surface L-selectin, while the p38MAPK inhibitor attenuated CLA-induced L-selectin shedding.

In summary, our studies demonstrated L-selectin expression was decreased to varying degrees in groups of varying severity patients with CLL and we explored a possible molecular mechanism that could lead to downregulation of this adhesion protein.

SUMMARY

Thrombotic or hemorrhagic events that occur during pathological processes may be due to increased or attenuated platelet function. The initial step of these processes is the ligand-receptor relationship, this provokes a signaling process that results in altered platelet activation. During this process, platelets come into contact with each other or further cellular elements in the circulation e.g. white blood cells, via different receptors. Cell surface receptors play an important role in these cellular relationships, the amount of receptors can change during a pathological process or during drug treatment on both platelets and white blood cells.

In our studies, we examined the effect of different forms of LPS, anti-PF4 / heparin autoantibody and TKIs through immune receptor mediated platelet activation and function and we determined the quantity and the modulation of an adhesion receptor, L-selectin on white blood cells in CLL.

In these studies we found that out of the various forms of LPS only Re-LPS activated platelets and it potentiated the platelet activating effect of TRAP in the costimulation. Our flow cytometric functional test showed, the anti-PF4/heparin autoantibody that formed during HIT enhances procoagulant platelet formation in a heparin-dependent manner. We verified that among TKIs, dasatinib inhibited platelet activation through the GPVI receptor, while nilotinib had no such effect and we highlighted that the process is regulated by inhibition of Lyn, Fyn, and Src kinases. In CLL we detected decreased L-selectin expression on malignant B cells, this value further decreased with the progression of the disease. We described a molecular mechanism with the results of our in vitro experiments where lower phosphatase activity resulted in elevated pp38MAPK levels and decreased L-selectin expression.

MAIN SCIENTIFIC RESULTS, OBSERVATIONS

- Re-LPS is able to activate platelets and potentiate the platelet activating effect of the PAR-1 agonist, these observations contribute to the development of a hypercoagulable state during sepsis caused by Gram-negative bacteria.

- The anti-PF4/heparin autoantibody enhanced platelet procoagulant activity in a heparin-dependent manner in the in vitro functional test, which was positively correlated with antibody titers in patients with HIT and positively correlated with an increased likelihood of thrombosis. The flow cytometric functional test reliably indicates the clinical outcome of HIT.

- Therapeutic concentrations of dasatinib inhibited platelet activation through GPVI receptor, which was associated with decreased collagen-induced platelet aggregation, ATP secretion, and coated-platelet formation.

- In addition to aggregating platelets, dasatinib also inhibited GPVI agonist-induced procoagulant platelets and clot retraction.

- Dasatinib acts on platelet function through inhibition of Lyn, Fyn, Src tyrosine kinases.

- The platelet dysfunction-inducing effects of dasatinib may play a role in the bleeding symptoms of patients taking dasatinib and may raise the need for drug discontinuation in case of an invasive procedure, while nilotinib did not affect platelet function.

- In CLL, L-selectin expression on malignant B cells is lower and soluble L-selectin level is higher compared to control, these changes were correlated with clinical stadium of the disease.

We detected lower total and PP2A activities and higher pp38MAPK levels in malignant B-cells compared to control B-cells, these are associated with lower cell surface TACE expression.

- In our in vitro experiments, pretreatment with phosphatase inhibitor (CLA) increased the level of pp38MAPK in malignant and control B-cells, which was associated with a decreased level of surface L-selectin. The p38MAPK inhibitor attenuated L-selectin decreasing effect of CLA in control B cells.

LIST OF PUBLICATIONS

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List of publications related to the dissertation

1. Mezei, G., **Bekéné Debreceni, I.**, Kerényi, A., Reményi, G., Szász, R., Illés, Á., Kappelmayer, J., Batár, P.: Dasatinib inhibits coated-platelet generation in patients with chronic myeloid leukemia.
   Platelets. 30 (7), 836-843, 2019.
   DOI: http://dx.doi.org/10.1080/09537104.2018.1501470
   IF: 3.106 (2018)

2. **Bekéné Debreceni, I.**, Mezei, G., Batár, P., Illés, Á., Kappelmayer, J.: Dasatinib Inhibits Procoagulant and Clot Retracting Activities of Human Platelets.
   Int. J. Mol. Sci. 20 (21), 1-14, 2019.
   DOI: http://dx.doi.org/10.3390/ijms20215430
   IF: 4.183 (2018)

3. **Bekéné Debreceni, I.**, Szász, R., Kónya, Z., Erdődi, F., Kiss, F., Kappelmayer, J.: L-Selectin Expression Is Influenced by Phosphatase Activity in Chronic Lymphocytic Leukemia.
   Cytom. Part B-Clin. Cytom. 96 (2), 149-157, 2019.
   DOI: http://dx.doi.org/10.1002/cyto.b.21771
   IF: 2.938 (2018)

4. Kerényi, A., **Bekéné Debreceni, I.**, Oláh, Z., Ilonczai, P., Bereczky, Z., Nagy, B. J., Muszbek, L., Kappelmayer, J.: Evaluation of flow cytometric HIT assays in relation to an IgG-specific immunoassay and clinical outcome.
   Cytom. Part B-Clin. Cytom. 92 (5), 389-397, 2017.
   DOI: http://dx.doi.org/10.1002/cyto.b.21362
   IF: 2.757

5. Kappelmayer, J., **Bekéné Debreceni, I.**, Vida, A., Antal-Szalmás, P., Clemetson, K. J., Nagy, B. J.: Distinct effects of Re- and S-forms of LPS on modulating platelet activation.
   J. Thromb. Haemost. 11 (4), 775-778, 2013.
   IF: 5.55

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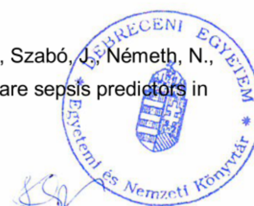
List of other publications

6. Orosz, Z. Z., Bárdos, H., Shemirani, A. H., **Bekéné** Debreceni, I., Lassila, R., Riikonen, A. S., Kremer Hovinga, J. A., Seiler, T. G., van Dorland, H. A., Schroeder, V., Boda, Z., Nemes, L., Früh Eppstein, B., Nagy, B., Facskó, A., Kappelmayer, J., Muszbek, L.: Cellular Factor XIII, a Transglutaminase in Human Corneal Keratocytes.
   Int. J. Mol. Sci. 20 (23), 5963, 2019.
   DOI: http://dx.doi.org/10.3390/ijms20235963
   IF: 4.183 (2018)
7. Gyöngyösi, A., Szőke, K., Fenyvesi, F., Fejes, Z., **Bekéné Debreceni, I.**, Nagy, B. J., Tósaki, Á., Lekli, I.: Inhibited autophagy may contribute to heme toxicity in cardiomyoblast cells.
   Biochem. Biophys. Res. Commun. 511 (4), 732-738, 2019.
   DOI: http://dx.doi.org/10.1016/j.bbrc.2019.02.140
   IF: 2.705 (2018)
8. Tóth, J., **Bekéné Debreceni, I.**, Deák, Á., Pető, K., Berhés, M., Hajdu, E., Szabó, J., Németh, N., Fülesdi, B., Kappelmayer, J.: Characteristics of thrombin generation in a fulminant in a fulminant porcine sepsis model.
   Thromb. Res. 158, 25-34, 2017.
   DOI: http://dx.doi.org/10.1016/j.thromres.2017.07.030
   IF: 2.779
9. Fejes, Z., Póliska, S., Czimmerer, Z., Káplár, M., Penyige, A., Gál Szabó, G., **Bekéné Debreceni, I.**, Kunapuli, S. P., Kappelmayer, J., Nagy, B. J.: Hyperglycemia suppresses microRNA expression in platelets to increase P2RY12 and SELP levels in type 2 diabetes mellitus.
   Thromb. Haemost. 117 (3), 529-542, 2017.
   DOI: http://dx.doi.org/10.1160/TH16-04-0322
   IF: 4.952
10. Hudák, R., **Bekéné Debreceni, I.**, Deák, I., Gál Szabó, G., Hevessy, Z., Antal-Szalmás, P., Osterud, B., Kappelmayer, J.: Laboratory characterization of leukemic cell procoagulants.
    Clin. Chem. Lab. Med. 55 (8), 1215-1223, 2017.
    IF: 3.556
11. Tóth, J., **Bekéné Debreceni, I.**, Berhés, M., Hajdu, E., Deák, Á., Pető, K., Szabó, J., Németh, N., Fülesdi, B., Kappelmayer, J.: Red blood cell and platelet parameters are sepsis predictors in an Escherichia coli induced lethal porcine modell.
    Clin. Hemorheol. Microcirc. 66 (3), 249-259, 2017.
    DOI: http://dx.doi.org/10.3233/CH-170271
    IF: 1.914



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12. Hudák, R., Vincze, J., Csernoch, L., **Bekéné Debreceni, I.**, Oláh, T., Erdődi, F., Clemetson, K. J., Kappelmayer, J.: The phosphatase inhibitor calyculin-A impairs clot retraction, platelet activation and thrombin generation.
    Biomed Res. Int. 2017, 1-10, 2017.
    DOI: http://dx.doi.org/10.1155/2017/9795271
    IF: 2.583

13. Becs, G., Hudák, R., Fejes, Z., **Bekéné** Debreceni, I., Bhattoa, H. P., Balla, J., Kappelmayer, J.: Haemodiafiltration elicits less platelet activation compared to haemodialysis.
    BMC Nephrol. 17 (1), 147, 2016.
    DOI: http://dx.doi.org/10.1186/s12882-016-0364-x
    IF: 2.289

14. Szűk, T., Fejes, Z., **Bekéné** Debreceni, I., Kerényi, A., Édes, I. F., Kappelmayer, J., Nagy, B. J.: Integrity bare-metal coronary stent-induced platelet and endothelial cell activation results in a higher risk of restenosis compared to Xience everolimus-eluting stents in stable angina patients.
    Platelets. 27 (5), 410-419, 2016.
    DOI: http://dx.doi.org/10.3109/09537104.2015.1112368
    IF: 2.465

15. Reményi, G., Szász, R., **Bekéné Debreceni, I.**, Szarvas, M., Batár, P., Nagy, B. J., Kappelmayer, J., Udvardy, M.: Comparison of coated-platelet levels in patients with essential thrombocythemia with and without hydroxyurea treatment.
    Platelets. 24 (6), 486-492, 2013.
    DOI: http://dx.doi.org/10.3109/09537104.2012.731112
    IF: 2.627

16. Nagy, B. J., **Bekéné Debreceni, I.**, Kappelmayer, J.: Flow cytometric investigation of classical and alternative platelet activation markers.
    EJIFCC. 23 (4), 124-134, 2013.

17. Antal-Szalmás, P., Nagy, B. J., **Bekéné Debreceni, I.**, Kappelmayer, J.: Measurement of soluble biomarkers by flow cytometry.
    EJIFCC. 23 (4), [1-8], 2013.

18. Miszti-Blasius, K., **Bekéné Debreceni, I.**, Felszeghy, S. B., 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

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19. Nagy, B. J., Szűk, T., **Bekéné Debreceni, I.**, Kappelmayer, J.: Platelet-derived microparticle levels are significantly elevated in patients treated by elective stenting compared to subjects with diagnostic catheterization alone.
Platelets. 21 (2), 147-151, 2010.
DOI: http://dx.doi.org/10.3109/09537100903477582
IF: 2.117

20. Simon, Z., Kiss, A., Erdődi, F., Setiadi, H., **Bekéné Debreceni, I.**, Nagy, B. J., Kappelmayer, J.: Protein phosphatase inhibitor calyculin-A modulates activation markers in TRAP-stimulated human platelets.
Platelets. 21 (7), 555-562, 2010.
DOI: http://dx.doi.org/10.3109/09537104.2010.499156
IF: 2.117

21. Ivády, G., **Bekéné Debreceni, I.**, Kissné, S. V., Hevessy, Z., Kappelmayer, J.: A timidin kináz aktivitás összehasonlító elemzése egyéb prognosztikai markerekkel krónikus lymphocytás leukémiában.
Klin. Kísérl. Lab. Med. 33 (2), 7-11, 2008.

Total IF of journals (all publications): 55,261
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