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

Examination of cellular procoagulant function by thrombin generation

by Renáta Hudák, MD

Supervisor: Prof. János Kappelmayer, MD, PhD, DSc

University of Debrecen
Doctoral School of Kálmán Laki
Debrecen, 2018

Examination of cellular procoagulant function by thrombin generation

By Renáta Hudák, MD

Supervisor: Prof. János Kappelmayer, MD, PhD, DSc

Doctoral School of Kálmán Laki
University of Debrecen

Head of the **Examination Committee**:	Prof. Zoltán Papp, MD, PhD, DSc
Members of the Examination Committee:	Péter Batár, MD, PhD
	Gábor Róbert Kiss, MD, PhD

The Examination takes place at the Library of the Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen at 11:00 am, 29th March, 2018.

Head of the **Defense Committee**:	Prof. Zoltán Papp, MD, PhD, DSc
Reviewers:	Prof. Barna Vásárhelyi, MD, PhD, DSc
	Lajos Gergely, MD, PhD
Members of the Defense Committee:	Péter Batár, MD, PhD
	Gábor Róbert Kiss, MD, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 13:00, 29th March, 2018.

INTRODUCTION AND REVIEW OF LITERATURE

Disturbances of the natural balance between the procoagulant and anticoagulant systems of the hemostasis - due to genetic or acquired factors - may result in bleeding or thrombotic diseases. Diseases that affect veins and/or arteries manifest as venous thromboembolism or pulmonary embolism are the leading causes of death worldwide. The enhanced activation of the hemostatic system can be caused by greater or longer than the physiologic activation of the cells in the circulation. The mediators that are secreted by the cells can also lead to enhanced activation of the hemostatic system.

Platelets have an essential role in the activation of the hemostatic system and the aberrant activation of platelets play a crucial role in the pathogenesis of atherosclerotic diseases. The events are triggered by disruption of the endothelium, plaque rupture or during interventions on coronaries, when platelets tether to surface-bound von Willebrand Factor (vWF), which initiates platelet activation and allows them to adhere to subendothelial components.

The aim of this study was to explore the effects of the phosphatase inhibitor calyculin A (CLA) on resting and activated platelets and to simultaneously investigate clot formation, platelet activation, and thrombin generation and their modulation in platelet rich plasma. Phosphatidylserine (PS)-expression, intracellular calcium responses using a novel cytosolic Ca^{2+} level measurement, clot retraction and thrombin generation were studied with or without TRAP activation.

It is known that malignant diseases can be associated with an increased risk of venous thromboembolism compared to the healthy population. This can be associated with solid tumors and hematological malignancies. Numerous factors may be involved in the development of thromboembolic events i) activation of the malignant cells, ii) the procoagulant proteins that are expressed on the cell surface or the shedding of procoagulant microparticles and iii) the production of interleukins, cytokines and other mediators. One type of acute myeloblastic leukemia (AML), the acute promyelocytic leukemia has long been described to cause activation of blood coagulation and thus can lead to disseminated intravascular coagulation, however, other AML types are lot less investigated in this respect. Aside from these well documented causes, acute leukemias can develop a hypercoagulable state, that frequently remains subclinical.

We hypothesized that monocytic leukemic cells have a higher procoagulant activity, thus we characterized their thrombin generating potential by different assays using coagulation factor deficient plasmas and TF neutralization. Results were compared to isolated normal human monocytes as negative- and to an acute promyelocytic leukemia cell line as positive controls.

The activation of the coagulation cascade in atherothrombotic status, the role of platelets

Platelets play a crucial role in physiologic hemostasis as well as in pathological conditions, platelets are important contributors of cardiovascular diseases underlying atherosclerosis. Pathological events are triggered by disruption of the endothelium, plaque rupture, when proteins of the subendothelial matrix (collagen, fibronectin, laminin) exposed, including the surface-bound von Willebrand Factor (vWF), which initiates platelet activation and allows them to adhere to subendothelial components. After adhesion platelets become activated, a shape change occurs subsequently procoagulant proteins are secreted from their intracellular granules and aggregation is triggered.

Ways of platelet activation

Different mediators can lead to platelet activation. Adenosine-diphosphate (ADP) activates platelets through their membrane $P2Y_{12}$ receptor. Thromboxane-A_2 has an important role in the formation of primer thrombocyte plug through the platelet thromboxane-A_2 receptors. Collagen activates the glycoprotein Ib (GPIb), glycoprotein IIb/IIIa (GPIIb/IIIa), glycoprotein Ia/IIa (GPIa/IIa) and glycoprotein VI (GPVI) receptors and provide thrombocyte-vWF to platelet-homotypic interactions. Protease-activated receptor 1 (PAR-1), the primary platelet thrombin receptor is G-protein-coupled. The activation of this receptor is the strongest platelet agonist and an important contributor to atherothrombotic processes. PAR-1 receptor activation via thrombin or relevant thrombin receptor activating peptides (TRAPs) results in a series of signaling events terminating in platelet shape change and granule secretion via the $G_{12/13}$ proteins and intracellular calcium release via the G_q mediated inositol triphosphate (IP_3) pathway. Intracellular IP3 receptors can be directly activated by pharmacological agents like thiomersal that has been used previously as a calcium mobilizer and cell function-modulating agent.

Activation of platelets is associated with the exposure of negatively charged phospholipids, which have high potential to bind coagulation factors and assemble enzyme-cofactor complexes that are crucially important for efficient propagation of the system. Under normal conditions, PS is present in the inner-layer leaflet of the plasma cell membrane.

Venous thromboembolic complications in malignancies

The clinical manifestations of hemostasis deregulation may vary from a subclinical asymptomatic hypercoagulable state to manifest thrombosis of the large vessels or, further, to systemic disseminated intravascular coagulation with severe bleeding. The risk of venous thromboembolism (VTE) in malignant cancer patient is 7-fold higher compared to the healthy population. Multiple clinical risk factors including the classical thrombosis risk factors (i.e. immobility, age, surgery-related) and the tumor specific factors (type of cancer, stage of cancer, chemotherapy) influence the risk of VTE. According to novel results, procoagulant factors (i.e. tissue factor) expressed on the surface of tumor tissue can activate the hemostatic system or other cells of the host. Microparticles, inflammatory cytokines (e.g. TNFα, IL-1β) shed from the intracellular granules, adhesion molecules and proangiogenic factors produced by the malignant cell or activated cell can contribute to the procoagulant effect. Platelets can be activated directly by the tumor cells or indirectly by agonist secreted by the tumor cells in the circulation. During activation, the platelet surface becomes procoagulant and promotes the the function of the coagulation cascade, while shedding of microparticles propagate the procoagulant effect.

Venous thromboembolic complication in malignant hematological diseases

In malignant hematological diseases, including acute leukemias the risk of VTE was considered low and rather bleeding complications were expected. Recent studies show that VTE risk in hematological malignancies is similar to that established in solid tumors. Numerous factors may be involved in the development of thromboembolic events in leukemias. Several of these cases may be related to therapeutic interventions like the central venous catheter associated prothrombotic effect or the therapy-associated thrombotic events of L-asparaginase treatment. One type of the acute myeloblastic leukemias (AML), the acute promyelocytic leukemia has long been described to cause activation of blood coagulation and thus can lead to disseminated intravascular coagulation. Other AML and ALL types are lot less investigated in this respect, although systemic coagulation activation has been detected in other types of AML and ALL as well.

The origin of microparticles and their procoagulant role

Microparticles (MP) range in size from 0.1-1.0 μm in diameter, formed from membrane blebs that are released from the cell surface by proteolytic cleavage of the cytoskeleton of apoptotic

or activated cells. Microparticles are present in the blood of healthy individuals and are increased in various diseases, including cardiovascular disease, diabetes, sepsis and cancer. In peripheral blood the number of the microparticles derived from platelets is the highest (80%), but microparticles derived from granulocytes, endothelial cells, red blood cells or monocyte can also found in the circulation. During the formation of MPs there is loss of membrane asymmetry with ionic phospholipids being transferred to the outer membrane of the MP. Importantly, the presence of PS significantly increases the procoagulant activity of MPs because it facilitates the assembly of components of the clotting cascade.

Thrombin generation test and its application

Thrombin is the key enzyme plays a pivotal role in clot promotion, it has numerous essential biological function, like the activation of thrombocytes, catalyzes the conversion of fibrinogen to fibrin and provides a positive feedback for the coagulation cascade. The formation of thrombin is one of the most important step of the coagulation process.

Conversion of fibrinogen to fibrin and formation of clot occurs when less than 5% of the total thrombin has been generated. Clot formation is the endpoint in standard clot-based assays, such as the prothrombin time (PT) and activated partial thromboplastin time (APTT), indicating that the PT and the APTT are determined by less than 5% of the thrombin generated in a sample. It means that most of the thrombin (>95%) is generated after the moment of clotting, therefore the clotting time is not automatically a relevant indicator of thrombin activity.

Thrombin generation test (TGT) is a global hemostasis test measures the amount and kinetic of generated thrombin in a time course. Originally the TGT was expanded for platelet poor plasma (PPP) examination. It is suitable for the measurement of platelet rich plasma and whole blood also. The *ex vivo* generated thrombin can be measured after the addition of a small amount of activator (trigger reagent) to the sample.

In our experiments the thrombin generation test was performed under modified conditions, as we were interested in cell-induced thrombin formation. The thrombin generation was measured in platelet rich plasma after activation and/or inhibition of platelets by adding 1 pM recombinant TF. In this model the phospholipid expressed on the surface of platelets provided the propagation in the model.

In the leukemia cell and leukemic MP studies we did not use any trigger reagent, the procoagulant proteins on the surface of the cells or microparticles provided the trigger for thrombin generation initiation and propagation.

OBJECTIVES

1. Effect of calyculin-A on platelet activation

Our aim was to explore the effects of the protein phosphatase inhibitor CLA on clot formation, platelet activation, and thrombin generation in platelet rich plasma. We hypothesized that CLA inhibits platelet activation the subsequent thrombin generation and influence clot structure. As a common mechanism, we postulated that CLA influences intracellular calcium levels.

2. The determination of procoagulant potential of acute monocytic leukemia cell lines

In our experiments we examined the procoagulant potential of three acute monocytic leukemia cell lines. Our hypothesis was, that the monocytic leukemia cell lines have a higher procoagulant potential compared to isolated normal human monocytes.

3. Examination of cellular models with thrombin generation test

Our aim was to examine thrombin generation after modulating platelet function in platelet rich plasma and to set up a model to determine the procoagulant potential of monocytes or monocytic leukemia cells.

MATERIALS AND METHODS

Preparation of platelet poor plasma

For each experiments, peripheral blood was drawn from 5 healthy volunteers - with no medications for at least 2 weeks prior to the experiments - in vacutainer tubes containing 0.105 M sodium citrate (Becton Dickinson, Franklin Lakes, NJ). Platelet poor plasma was separated by centrifugation at 1220 g for 15 min, room temperature according to the protocol that used in our routine diagnostic laboratory. Pooled platelet rich plasma was prepared by adding 1-1 mL of platelet poor plasma of each individuals. Platelet poor plasma is called hereafter normal plasma.

Preparation of platelet rich plasma

Whole blood was drawn from healthy volunteers with no medications for at least 2 weeks prior to the experiments. Blood samples from volunteers was drawn in vacutainer tubes containing 0.105 M sodium citrate (Becton Dickinson, Franklin Lakes, NJ). Platelet rich plasma (PRP) was prepared from venous whole blood by centrifugation at 170 x g for 15 minutes at room temperature (RT). Platelet count of PRP was adjusted to 250 G/L by adding platelet poor plasma (PPP). PPP was obtained by centrifugation of the citrated blood sample at 1500 x g for 15 minutes at RT. In subsequent experiments the following PRP samples were analysed:

1. Non-activated sample (NA)
2. CLA-pretreated non activated (NA+CLA)
3. Thrombin receptor activating peptide (TRAP)-activated sample (TRAP)
4. CLA-pretreated TRAP-activated (TRAP+CLA).

In preliminary experiments 50 nM CLA was found to inhibit degranulation of platelets as well as PAC-1 binding without being toxic to cells so this concentration of CLA was applied in the experiments. TRAP (Sigma-Aldrich, St. Louis, MO) was used at a final concentration of 20 μM.

Isolation of normal human monocytes

Hundred sixty mL of EDTA-anticoagulated (K_2EDTA, Becton Dickinson vacutainer tube, San Jose, CA, USA) peripheral blood was drawn from healthy volunteers, and peripheral blood mononuclear cells (PBMC) were separated on Ficoll Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA). Normal human monocytes were isolated from PBMC by a magnetic monocyte separation, negative isolation kit (Dynabeads® Untouched Human Monocytes, Invitrogen by Life Technologies) using beads in combination with an antibody mix (contains biotinylated mouse IgG antibodies for CD3, CD7, CD16 (specific for CD16a and CD16b), CD19, CD56, CDw123 and CD235a - Glycophorin A -) to remove all unwanted cells from the sample, leaving only the monocytes behind. This technique – unlike other column based methods – provides a 95% viability that is important from the point of view of functional tests like thrombin generation. The cell count was adjusted to 5 x 10^6/mL in phosphate-buffered saline (PBS) for thrombin generation measurements and 10^7/mL for flow cytometric analysis. Normal human monocytes were used as negative control in our leukemic cell experiments.

Cell cultures and culturing

The THP-1 cell line was a gift of prof. Dr. György Vereb (Department of Biophysics and Cell Biology, University of Debrecen, Hungary). MOLM-13 and MV4-11 cell lines were purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Based on the WHO 2008 criteria for the stratification of AML, all these cell lines belong to the unfavorable prognostic group with multiple chromosomal abnormalities. As a positive control an acute promyelocytic leukemia cell line (NB4, which was a gift of prof. Dr. Zoltán Balajthy, Department of Biochemistry and Molecular Biology, University of Debrecen, Hungary) was used. All cell lines were maintained in suspension cultures, in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO), supplemented with 15% fetal bovine serum (heat inactivated, Sigma-Aldrich, St. Louis, MO) were grown in a cell incubator at 37ºC in a humidified atmosphere of 5% CO_2 in air. The duplication times of monocytic leukemia cells were 35-48 hours which was investigated by cell counting at 48 hours and cell cycle analysis at 0, 24 and 48 hours. The medium was changed three times a week, when maximum cell density was set to 0.5 x 10^6/mL in case of monocytic leukemia cell lines and to 0.3 x 10^6/mL in case of the NB4 cell line. Cell cultures were subcultured by total medium replacement by using centrifugation every seven days. Cell viability was >90%, evaluated by trypan blue exclusion before each experiment. Upon centrifugation (190 g, 25°C, 10 minutes) the leukemic cells were resuspended in PBS at a concentration of 5 x 10^6/mL for the functional thrombin generation test and to 10^7/mL for flow cytometric analysis.

Leukemic cell immunophenotype characterization by flow cytometry

For immunophenotyping surface staining of cells were carried out according to standard 8-color procedures. Fifty microliters of cell suspension adjusted to cell count of 10x10^9/L by PBS was incubated by saturating concentrations of directly conjugated antibodies for 15 minutes at room temperature in the dark with antibodies against different cell surface and cytoplasmic epitopes. Labelling was carried out with CD14-FITC, CD11b-PE, HLA-DR-PerCPCy5.5, CD4-PB, CD15-FITC, CD34-PerCPCy5.5, CD71-FITC, CD117-PE, CD38-APCH7 (Becton Dickinson Biosciences, San José, CA), CD34-APC, CD13-PECy7, CD64-APCAF750, CD33-PECy5.5, CD56-PECy7 (Beckman Coulter, Marseille, France), CD45-PO (Invitrogen, Thermo Scientific Inc., Walthman, MA), MPO-PE (Dako Cytomation, Glostrup, Denmark), and FXIII-A-FITC. PFA-fixed samples were kept at 4°C for maximum 24 h. Flow cytometric measurements were

carried out on a FACSCanto II (Becton Dickinson Biosciences, San Jose, CA) flow cytometer using the same setting for all investigated samples. Data obtained on 50 000 cells were acquired and stored in list-mode data files. Data were analyzed by FACSDiva software (Becton Dickinson Biosciences, San José, CA).

Determination of thrombocyte surface phosphatidylserine expression by flow cytometry

Platelet rich plasma (PRP, 110 µL) - using 250 G/L thrombocyte count - was pre-incubated with either HEPES buffer (15 mM NaCl, 25 mM HEPES, pH 7.4) containing 0.5% dimetilsulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) as control or the protein phosphatase inhibitor CLA, for 30 minutes at 37ºC in a water bath. CLA was used at a final concentration of 50 nM. After pre-incubation, platelets were activated either by TRAP at a final concentration of 20 µM or by thiomersal at a final concentration of 200 µM for 15 minutes at 37ºC in a water bath. Then PRP (5 µL) was stained with 5 µL monoclonal CD41-PE antibody, 5 µL Annexin-V-FITC and Annexin V binding buffer (1x concentrate) was added to examine the PS-expression of the platelets. In each experiment 10 000 events were collected in the platelet gate, measured by an FC500 flow cytometer and results were analysed with the Kaluza software (Beckman Coulter, CA).

Determination of phosphatidylserine and tissue factor expression of leukemic cell lines by flow cytometry

For the analysis of PS-exposure of monocytes and cell lines we used FITC-labelled Annexin V and Annexin V Binding Buffer from Becton Dickinson Biosciences, San José, CA. Tissue factor expression was detected by a monoclonal PE-conjugated anti-TF antibody (R&D Systems, Minneapolis, MN). 100 µL isolated monocytes or leukemic cells (1 x 10^6 cell/100 µL) were stained with10 µL TF-PE and 5 µL Annexin-V-FITC antibody for 30 minutes, at room temperature in dark. Then they were washed twice with PBS and cells were resuspended in 500 µL Annexin V Binding Buffer. In each experiment 30000 events were collected by an FC500 flow cytometer and results were analysed with the Kaluza software (Beckman Coulter, Miami, FL).

Microparticle separation and microparticle number determination by flow cytometry

First, 3.0 x 10^6 of cells were centrifuged at 190 g, 25°C for 10 minutes and the supernatant was discharged, cells were resuspended in fresh RPMI-1640 supplemented with 15% fetal bovine serum to adjust their cell count to 0.5 x 10^6/mL in case of monocytic leukemia cell lines and to 0.3 x 10^6/mL in case of NB4 cell line. The cells in suspension were cultured for 48 hours and the cell count was set to 5 x 10^6/mL. Cells were removed from the suspension by centrifugation (190 g, 25°C for 10 minutes) followed by a brief removal of residual intact cells from the supernatant by spinning at 13 000 g for 2 minutes at room temperature (RT). Subsequently, microparticles were isolated by a high speed centrifugation (16 100 g, 30 minutes, RT), washed with PBS and centrifuged (16 100 g, 30 minutes, RT). MPs were resuspended in 1 mL PBS. Prior to MP measurements, counting calibration beads were used for cytometer settings. Megamix-Plus SSC (Biocytex, Marseille, France) was used for this purpose, which is a mix of fluorescent beads of variable diameter selected to cover a major part of the microparticle size range, using SSC as a size-related parameter. The flow cytometer has been calibrated by these Megamix beads and we found that by the suggested marker (SSC) the FacsCanto II (Becton Dickinson, San Jose, CA) was capable of detecting beads in the range of 0.16 – 1.0 μm. MP number was evaluated by PS-positivity as measured on the cytometer.

Determination of procoagulant activity (PCA) of the leukemic cell lines

In the PCA tests 100 μL of normal or factor VII (FVII) deficient (Siemens, Marburg, Germany) or factor XII (FXII) deficient plasma (Siemens, Marburg, Germany) was incubated with 100 μL of PBS, monocytes or leukemic cells at 37°C for 3 minutes. Leukemic cell procoagulant activity was also measured after blocking the surface TF with a human anti-tissue factor antibody. Leukemic cell were incubated with a goat polyclonal IgG anti human-antibody (final concentration of 25 μg/mL, R&D Systems, Minneapolis, MN) for 15 minutes. 100 μL of blocked leukemic cells was incubated with 100 μL of normal plasma at 37°C for 3 minutes. As a negative control for the blocked sample we used goat serum at a final concentration of 45 μg/mL (Sigma-Aldrich, St. Louis, MO). Than 100 μL of 20 mM $CaCl_2$ was added to initiate clotting. Fibrin formation in seconds was measured by a KC-1 mechanical coagulometer. A 1:10 dilution of recombinant thromboplastin (Dade Innovin, Siemens, Marburg, Germany) was arbitrarily taken as 12 800 mU and a serial dilution was used to generate the thromboplastin

calibration curve on a double logarithmic scale. The clotting times were converted to procoagulant activity expressed as mU/10^5 cells.

Clot retraction analysis

PRP (720 µL) was preincubated with buffer control or CLA, then activated by TRAP. In a glass tube, 800 µL of each four samples were incubated with $CaCl_2$ (at a final concentration of 25 mM) for 60 minutes at 37ºC in a water bath. At time points 0, 20, 40 and 60 minutes, photos were taken to document clot formation. At the end of the experiment, the volume of the extruded serum was determined by an analytical balance. The amount of fibrin monomer in the extruded serum was measured by a latex enhanced quantitative immunoassay (Stago, Asniére, France) on the ACL-TOP coagulation analyser (Instrumentation Laboratory, Bedford, MA).

Procoagulant effect of leukemic cells and their microparticles determined by thrombin generation test

Thrombin generation test (TGT) was performed as described previously using the Thrombinoscope CAT (Calibrated Automated Thrombogram, Maastricht, The Netherlands) assay according to the manufacturer's instructions (Diagnostica Stago, Asnières, France). Briefly, 80 microliters of normal plasma/VII-deficient plasma/XI-deficient plasma/XII-deficient plasma was incubated with 20 µL of monocyte or leukemic cell for 10 minutes in round-bottomed 96-well black microplates in 37°C. In microparticle measurements 80 microliters of normal plasma was incubated with 20 µL of microparticles. For each sample, a calibrator (Thrombin Calibrator™) was run in parallel in order to correct the fluorescence signal for substrate consumption and plasma color variability. Thrombin generation was initiated by the addition of 20 µL of FluCa-Kit™ (a mixture of Fluorogenic substrate and Fluo-Buffer containing $CaCl_2$). All samples were run in duplicates. Fluorescence was detected by a Fluoroskan Ascent® fluorimeter (Thermo Fischer Scientific, Waltham, MA) and the thrombin generation curves were analysed by the Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands). Thrombin generation curves were characterised by the following parameters (calculated and presented by the Thrombinoscope software):

1. lagtime: the moment at which thrombin generation starts
2. endogenous Thrombin Potential (ETP): the area under the curve
3. peak Thrombin: the highest thrombin concentration
4. time to Peak: the time until the Peak Thrombin

5. start Tail: the time to end-point of thrombin generation
6. velocity Index: the slope of the curve between the beginning of thrombin generation and the Time to Peak parameter.

Determination of platelet function modulation and CLA effect on thrombin generation

Eighty microliters of pretreated PRP was incubated with 20 µL of standard preparations of 1 pM recombinant tissue factor (rTF, PRP-Reagent, Thrombinoscope BV, Maastricht, The Netherlands) for 10 minutes in round-bottomed 96-well black microplates. Thrombin generation was initiated by the addition of 20 µL of a mixture of fluorogenic substrate and Fluo-Buffer that contained $CaCl_2$ (Thrombinoscope BV, Maastricht, The Netherlands). Fluorescence was detected by a Fluoroskan Ascent® fluorimeter (Helsinki, Finland) and the thrombin generation curves were analysed by the Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands).

High-speed confocal measurement of platelet cytosolic Ca^{2+} levels

For measurements of intracellular Ca^{2+} levels, PRP was prepared as described above and RGDS peptide was added to prevent aggregation. PRP (100 µL) was loaded with 1.5 µL of 2 mM Fluo-4-AM for 10 minutes at 37ºC in a water bath. The cells were co-loaded with Fura-Red by addition of 2 µL of 1 mM Fura-Red-AM for 30 minutes at 37ºC in a water bath. Before centrifugation, 2 mL of HEPES-buffered saline was added and after centrifugation at 350 x g for 20 minutes at RT, the co-loaded platelets were collected and resuspended in 100 µL of the same buffer.

Platelets loaded with the calcium-indicator dyes were imaged using a Zeiss LSM 5 LIVE (Carl Zeiss AG, Jena, Germany) high-speed confocal scanning unit with a 40x oil immersion objective (NA: 1.3). The final 100 µL solution was pipetted onto a glass coverslip secured above the objective within a temperature-controlled chamber and was allowed to rest for 2 minutes before the measurement. Both the chamber and the objective had been preheated to 37 ºC and this temperature was kept constant during the measurement. Frames (x-y images) were recorded at a rate of 5 Hz for 5 minutes using 488 nm excitation wavelength and two detection channels: a band pass filtered channel between 500 and 525 nm for the Fluo-4 signal and a long pass filtered channel above 635 nm for the Fura-Red signal. The wide gap between the cutoff wavelengths ensured that there was minimal crosstalk between the channels. TRAP was added

by pipetting 4 µL of 500 µM TRAP solution to the platelet suspension on the coverslip. Measurement was continuous during the addition of TRAP. The confocal microscopic measurement and the evaluation of fluorescence signals were performed by János Vincze, MD from the Department of Department of Physiology, University of Debrecen.

Statistical analysis

The statistical analysis was performed with GraphPad Prism 5.0 version. Normality of the data was evaluated by the Kolmogorov-Smirnov and Shapiro-Wilk tests. Elements outside mean ± 2SD were removed as outliers. Data are expressed as mean ± SD or mean ± SEM. Predetermined pair-wise differences were analysed by paired t test and in case of a non-normal distribution, the Wilcoxon matched paired test was used. For non pairwise comparison, unpaired t test (Gaussian distribution) and Mann-Whitney test (non Gaussian) were used. ANOVA with Bonferroni post test (Gaussian distribution) or Kruskal-Wallis test with Dunn's post test (non Gaussian distribution) were used for multiple group comparison. *P* values less than 0.05 were considered as statistically significant.

RESULTS

The effect of TRAP and thiomersal activation and CLA inhibition on the surface PS-expression and microparticle formation of platelets

In a series of experiments, we determined the level of PS-exposure to analyse the effects of CLA on resting and activated platelets. PRP samples were preincubated with HEPES buffer (control) containing 0.5% DMSO or 50 nM CLA in DMSO for 30 minutes. Platelets were identified according to their CD41-PE staining then PS-expression was determined by Annexin-V positivity. The PS-expression of non-activated (NA) samples was low (mean ± SD=3.03 ± 0.84) and it was further decreased by CLA pretreatment (mean ± SD=1.75 ± 0.67, p=0.004). The PAR-1 receptor agonist, TRAP significantly increased the number of platelets expressing PS (mean ± SD=18.7 ± 3.23) compared to the NA sample ($p<0.0001$) while CLA pretreatment completely prevented PS upregulation in TRAP-stimulated platelets ($p<0.0001$). Contrary to the TRAP activation, thiomersal activation induced 96% PS-expression that could not be prevented by CLA-preincubation. CLA-pretreatment completely decreased the number of microparticle events in NA and TRAP-activated samples.

The effect of CLA on clot retraction

It could be observed that clot formation and retraction commenced already by 20 minutes and by 60 minutes both the non-activated and the TRAP activated samples displayed an intense clot retraction. The CLA-pretreated samples however were much less retracted. In accordance with this morphology the CLA pretreated samples showed a tendency for extruding less serum (NA: 754.4 mg vs. NA + CLA: 653.2 mg, p=0.0085). The fibrinogen concentration was unmeasurably low in the samples by 60 minutes, nevertheless the fibrin monomers (FM) were determined by a quantitative assay and it was found that in the extruded sera of CLA-pretreated samples more FM was detectable compared to non-pretreated samples.

Thrombin generation of platelet rich plasma samples after modulation

The thrombin formation was faster, the lagtime (NA: mean ± SD: 8.1 ± 1.1 min; TRAP: mean ± SD: 6.5 ± 1.5 min) and time to peak (NA: mean ± SD: 18.2 ± 2.8 min; TRAP: mean ± SD: 11.9 ± 1.5 min) were shorter in case of TRAP activation compared to the non-activated sample. TRAP activation increased the peak thrombin and also the velocity index. CLA preincubation significantly prolonged the time of thrombin generation in TRAP activated samples, but did not have significant effect on the NA sample. CLA attenuated the peak thrombin and velocity index already in NA samples and completely blocked the TRAP-elicited augmentation in these parameters.

The effect of CLA inhibition and TRAP activation on the intracellular Ca^{2+} level

First, all NA and CLA-pretreated platelets were observed for at least 60 seconds, than TRAP was added to the sample. Platelet intracellular Ca^{2+} levels were measured after activation of non-activated or CLA pretreated sample. Two typical patterns of intracellular calcium concentration changes of platelets were observed on TRAP-activated samples without CLA pretreatment: no transient change after the addition of 20 μM TRAP or a transient increase in the Ca^{2+} level. In the case of samples that were not pretreated, 22.2 % of platelets showed transient cytosolic Ca^{2+} increase upon TRAP addition. In case of platelets preincubated with calyculin-A, none of the investigated 122 cells showed calcium transients after TRAP administration during the time course of the measurement providing evidence for CLA blockage of Ca^{2+} elevation

Thiomersal evoked a prolonged elevation of cytosolic calcium in all the platelets followed by sustained store-operated calcium entry. Thiomersal induced cytosolic calcium elevation could not be blocked by CLA.

Immunophenotype and genetic characteristics of leukemic cell lines

THP-1 is a monocytic leukemia cell line derived from a patient at relapse, MOLM-13 is a secondary monocytic leukemia developed from myelodysplasia RAEB and MV4-11 is a de novo monocytic leukemia cell line. The myeloid marker CD33 was the only antigen that was uniformly expressed on the surface of all cell lines, while the other pan-myeloid marker CD13 as well as various monocytic markers displayed a variable expression. The three cell lines showed different immunophenotype with incongruent expression of monocytic and myeloid markers, however, THP-1 seemed to be the most mature with no CD34 and small CD117 expression. Complex cytogenetic abnormalities are present in all cell lines that are responsible for their poor prognosis, irrespectively of the FLT3-ITD mutation status.

Phosphatidylserine (PS) and tissue-factor (TF) expression of normal monocytes and leukemic cell lines

In case of non-activated normal human monocytes the TF-expression was high (mean ± SD: 73.4 ± 20.3%), while the TF-expression was different in the cell lines. The TF-expression of THP-1 cell line (mean ± SD: 73.1 ± 11.6%) was not different compared to normal monocytes. MOLM-13 cell line showed the lowest TF positivity (mean ± SD: 3.9 ± 0.6%). The TF-expression of MOLM-13 and MV4-11 (mean ± SD: 21.2 ± 7.4%) cell lines were significantly lower compared to monocytes, THP-1 and NB4 cell lines ($p<0.001$).

Among the three monocytic leukemia cell lines, the lowest PS-expression was observed on THP-1 cells (mean ± SD: 6.5 ± 2.3 %) and was somewhat higher on the MOLM-13 (mean ± SD: 12.1 ± 3.3 %) as well as on the MV4-11 cell line (mean ± SD: 21.8 ± 8.4 %) although differences were non-significant. There was, however, a significant difference in the intensity of PS-expression as characterized by the ratio of mean fluorescence intensity (MFI) values of the PS positive population divided by the MFI value of the unlabeled cells. The mean ± SD of the MFI ratio from 5 experiments was 14.12 ± 3.9 for THP-1 cells that was non significantly different from MV4-11 cells (mean ± SD: 20.23 ±12.12) but in case of MOLM-13 cells this ratio was significantly higher than in the other two cell lines (mean ± SD: 75.91 ± 30.22). This

means that although MV4-11 cells expressed the highest percentage of PS positivity, the intensity of expressed PS was highest on the MOLM-13 cell line.

Determination of microparticle number

By using the same cellular density for culturing during a 48-hour period all three cell lines generated considerable amount of MP. We isolated these MPs and investigated their procoagulant activity by TGT. We found that the THP-1 cell line produced twice as much MP as the other two cell lines. The mean MP number was $4.7x\ 10^7/5\ x\ 10^6$ cells vs. 2.2 and 2.6 x $10^7/5\ x\ 10^6$ cells in the MOLM-13 and MV4-11 cell lines respectively. The MP number of the positive control NB4 cell line was between these values ($3.3x\ 10^7/\ 5\ x\ 10^6$ cells). There was no significant difference among the number of microparticles shed by the different cell lines.

Procoagulant activity measured by a one-stage clotting assay (PCA)

The clotting time of normal plasma containing PBS but no cells was over range (> 200 sec, data not shown) and similar values were observed using isolated normal human monocytes, both referring to a very low procoagulant activity. Using the same cell concentrations, all three cell lines displayed significantly shorter clotting times and consequently higher procoagulant activity. The neglectable PCA of isolated normal human monocytes was unaffected by FVII deficient plasma. We observed that the FVII deficient plasma and a polyclonal anti-human tissue factor (ahuTF) antibody pretreatment completely abolished the fibrin forming capacity of all monocytic leukemia cell lines resulting in very low procoagulant activity, while FXII deficient plasma had no significant blocking effect. Although, the procoagulant activity of the acute monocytic leukemia cell lines was significantly increased compared to the buffer control or monocytes, there was no significant difference between the three cell lines in normal plasma.

Thrombin generation of leukemic cell lines

Another way to evaluate the procoagulant activity of cells was done by the thrombin generation test. The positive control NB4 cell line resulted in an abrupt increase in thrombin and reached the highest Peak thrombin value. The monocytic leukemia cell lines (THP-1, MOLM-13 and MV4-11) displayed rather similar thrombin generation profiles and thrombin was formed much faster than in case of normal human monocytes.

There was no significant difference between the thrombin generation of normal monocytes compared to PBS in normal plasma. Intact leukemic cells of all three cell lines significantly

shortened the lagtime and time to peak, the shortest times were observed in the positive control NB4 cells. FVII deficient plasma and anti-TF pretreatment completely eliminated the shortenings in the monocytic leukemia cells, while FXI and FXII deficient plasma had no such effect. In case of the Time to Peak parameter the TF-blocked and FVII deficient samples exerted the same effect on THP-1 cells, but attenuated neutralizing effects were observed on the two other cell lines. Importantly, on the MOLM-13 cell line and to a smaller extent in case of MV4-11 cells, FXI deficiency also prolonged the Time to Peak similarly to TF blockage while FXII deficient plasma did not significantly affect this parameter. We found that healthy monocytes generated the same low amount of thrombin as normal plasma without any cells, while all monocytic leukemia cell lines induced considerable thrombin generation as observed in the Peak thrombin parameter, however, it did not reach the level of the promyelocytic leukemia NB4 cells. The elevation in the quantity of produced thrombin was non-significant in case of THP-1 cells compared to normal monocytes but peak thrombin values were significantly higher than the monocyte control in case of the MOLM-13 and MV4-11 cell lines. The peak thrombin parameter was dependent on factors VII, XI and XII in case of all cell lines, however, a nonsignificant FVII dependence was observed in case of MOLM-13 cells. Factor VII deficiency affected nearly all investigated parameters, while eliminating factor XI from the system caused a considerable reduction in thrombin generation that was particularly striking in case of MOLM-13 and MV4-11 cells. Interestingly, anti-TF pretreatment - with the antibody that effectively blocked the shortening of lagtime - was ineffective in modulating the formed thrombin quantity.

Thrombin generation of microparticles

By using the same cellular density for culturing during a 48-hour period all three cell lines generated considerable amount of MP. We isolated these MPs and investigated their procoagulant activity by TGT. These MP have strong thrombin inducing ability as demonstrated by the significantly reduced lagtime and time to peak values, compared to a buffer control (PBS). No significant differences were observed in peak thrombin and velocity index parameters.

DISCUSSION

Possibilities of platelet function modulation

Platelets have an essential role in the pathogenesis of arterial atherosclerotic diseases. For the investigation of modulation of platelets, we set up an *in vitro* 'cellular' model. In our experiments we studied the platelet activation according to PS-expression, and investigated their effect on coagulation and thrombin generation with functional tests (clot retraction analysis, thrombin generation test). Furthermore, we examined the effect of the protein phosphatase inhibitor, CLA on platelet PS-expression, function and intracellular Ca^{2+} level.

In our experiments instead of thrombin we utilized TRAP - a potent activator of PAR-1 on platelets - to avoid early clotting and any potential pleiotropic effects of thrombin.

Serine/threonine protein phosphatases play an essential role in cells, including platelet signalling, metabolism, platelet secretion and initiation of aggregation. Under experimental conditions in phosphatase-inhibitor treated platelets, actin polymerization was inhibited, microtubules were reorganized in sustained pseudopods, and the phosphorylation of myosin light chain was not enhanced upon thrombin stimulation. The myosin phosphorylation is crucial in actomyosin contraction for platelet secretion and activation.

Activation of platelets by various agonists is accompanied by a transient intracellular Ca^{2+} elevation, which is obligatory for the initiation and propagation of platelet responses such as adhesion, aggregation and degranulation. The rise in intracellular calcium is a result of the calcium release from the intracellular stores mediated by the IP_3 receptor. In earlier studies, it was found that CLA treatment of activated platelets suppressed the activation induced rise in Ca^{2+} levels measured by fluorescence spectrophotometric techniques. We have investigated platelet cytosolic Ca^{2+} levels by a real-time ratiometric measurement with confocal microscopy, to more appropriately characterize changes in intracellular calcium signals. It was found that activating platelets by TRAP, causes a clear intracellular Ca^{2+} signal in 22.2% of platelets that was abolished by CLA-pretreatment and this effect was not due to affect the resting calcium levels.

In control samples, upon TRAP activation (in non-CLA pretreated samples), a heterogeneous response was predictable there is current evidence for platelet subpopulations. These platelets were described as procoagulant platelets, while the remaining population of platelets can be considered as contracting platelets where the GPIIb/IIIa activation is observed. Heterogeneity of platelet responses and functions are important in the thrombus-forming process. After

platelet activation, distinct platelet populations may have different roles in the coagulation process, depending on their activation state and surface properties. Similarly, to calcium signals, not all platelets express PS upon activation,

In our experiments, we also observed that only 20% of platelets responded by PS-expression upon TRAP-stimulation a similar ratio observed in case of calcium transients. By using thiomersal, a direct activator of the intracellular IP3 receptors 96% of platelets showed PS-positivity that could not blocked by CLA. We could verify both by flow cytometric PS-expression studies, as well as by the ratiometric calcium measurement, that PP inhibition by CLA does not impair direct IP3 receptor stimulation, so its effect is exerted upstream of the IP3 receptor.

Raised Ca^{2+} levels lead to the exposure of PS on the platelet surface that serves as a site for the assembly of intrinsic and extrinsic tenase and prothrombinase complexes, thus it is critical for thrombin generation in PRP. The speed of thrombin generation and the activity of generated thrombin were shown to be the most useful parameters to evaluate the thrombin generation of PRP. CLA inhibited TRAP-elicited thrombin generation and we demonstrated that CLA also inhibited that platelet subpopulation which takes part in the clot retraction.

Taken together, these findings indicate that inhibition of protein phosphatases by CLA-pretreatment can be regarded as a useful tool to investigate the platelet subsets that contribute to enhanced thrombin formation and clot retraction.

Enhanced procoagulant activity of acute monocytic leukemia cell lines

In the initiation of coagulation, the key surface protein is TF that binds factor VIIa and rapidly leads to the activation of the extrinsic coagulation cascade, leads to thrombin generation and fibrin formation. According to previous concept TF is not present in the circulating blood, it can be found under the endothelial layer in the extravascular space. In earlier studies we have observed a considerable difference in tissue factor activity of activated intact and lysed monocytes, as the latter displayed a much higher procoagulant activity and similar differences between intact and lysed cells have been described more recently for PBMC samples derived from patients suffering from AML. Using lysed cancer cell lines, some authors found FVII-dependence of the promyelocytic NB4 cell line and the MCF7 breast cancer cell line, but they detected very low thrombin generation, with no TF activity and no FVII-dependence in other leukemia cell lysate like the K562 chronic myeloid leukemia and the HEL cell erythro-leukemia cell lines.

According to novel experiments, in physiological conditions, the monocytes the only cells in the circulation that can synthetize TF and upon activation monocytes can express it - in a significant amount - on their surface, which could participate to coagulation.

Isolated, non-activated monocytes used as negative control in our experiments, 73% of them expressed TF on their surface but no functional role was detected during the functional procoagulant activity and thrombin generation tests. The thrombin generation capacity of these monocytes was the same as the control sample without cells. It is known that TF can appear in two forms on the surface on vascular cells. It can be as a functionally inactive form, 'cryptic' or it can be active, 'decryptic'.

Our monocytic leukemia cell lines showed different TF expression, but there was no significant difference between the procoagulant effect of the acute monocytic leukemia cell lines in normal plasma, measured by functional tests.

According to recent results, different ways can lead to TF activation. The majority of the evidence in the literature point out that exposure of anionic phospholipids, such as PS, on the outer leaflet of plasma membrane is a key regulator of TF procoagulant activity at the cell surface. There was no significant difference found between the PS-expression of our acute monocytic leukemia cell lines, but the intensity of PS-expression in case of the MOLM-13 cell line was significantly more intense than the intensity of the THP-1 and MV4-11 cell lines.

Leukemic cells displayed significantly higher procoagulant activity compared to normal monocytes. We supposed that various pathways are involved differently in this process, therefore we investigated the role of FXII and FVII that are essential in the initiation of the intrinsic and extrinsic coagulation pathway. In a one-stage clotting assay very little thrombin is sufficient to form a clot in the coagulometer within a few minutes as a result of the formed fibrin mesh. This process seems to be totally FVII-dependent as was exemplified by the parallel abrogating effect of VII-deficient plasma and tissue factor blocking antibody.

In the thrombin generation assay, however the reaction is followed up to 1 hour, well beyond that of fibrin formation, enabling one to better characterize the contribution of individual coagulation factors by using factor deficient plasmas. Based on our observations we propose that FVII is important in triggering the procoagulant effect evidenced by the fibrin formation and lagtime in the TGT. Subsequent thrombin generation as determined by the time to peak and peak thrombin, however, is very much dependent on factor XI and partially on FXII as these proteases seem to be necessary in the propagation of the coagulant reaction. The particular role of FXI in peak thrombin for the MOLM-13 and to some degree the MV4-11 cells suggest that the thrombin generation is mediated by a feedback activation of FXI by thrombin at least

partially independently of FVII and FXII. This is in agreement with the role of FXI in affecting the time to peak by MOLM-13 and partially for MV4-11 cells. The discrepancy between the effect of using FVII deficient plasma or anti-TF antibody in peak thrombin measurement for THP-1, MOLM-13 and MV4-11 cells might stem from a TF-independent activation of FX/FIX by FVII and PS exposed on the cells and a resultant feedback activation of FXII by thrombin.

It was described that in several disorders microparticles shed by endothelial cell or blood cells display a procoagulat effect and that this can influence intravascular coagulation. We supposed that microparticles from our leukemic cell lines have an enhanced procoagulant effect. We have found that an intact cell produces 4-10 microparticles in a 48-hour period.

The overall contribution to the generated thrombin depends on the surface of the particle and its potentially procoagulant receptors. If we consider leukemic monocytes and their microparticles as spheres, their surface can be calculated. Taking into consideration the detection limit of the Canto-II flow cytometer that was used for the analysis of microvesicles and monocytic cells we can estimate the radii of the MP and cells at 0.3 μm and 10 μm respectively. Based on the formula for the calculation of the surface for spheres ($A = 4\ r^2\ \pi$) the intact cells possess about a 1000-fold larger surface than that of the microparticles. Since during a 2-day culturing, 4-10 microparticles were released from one intact leukemic cell, this would imply that per a given surface, MP derived from these monocytic leukemia cells are roughly a 100-fold more procoagulant than their parent cells. All MP contribute to thrombin generation mostly by reducing the lagtime and to a much lesser extent the time to peak parameters. This means that MP derived from these monocytic cell lines rather affect the initiation- and not the propagation phase - of thrombin formation.

In these experiments we have found, that monocytic leukemia cell lines and MP generated by these cells exhibit significantly higher procoagulant activity compared to normal monocytes and that this effect is primarily TF dependent, however, other factors may modulate this effect. Our results demonstrate that functional assays may provide a useful tool to identify cell induced thrombin generation in acute myeloid leukemias.

SUMMARY

We studied the effects of the protein phosphatase inhibitor, Calyculin-A on platelet activation, clot formation and thrombin generation. We found that CLA completely blocked the PS-expression of platelets in both the non-activated and the TRAP-activated platelet rich plasma sample. CLA also inhibited the process of thrombin generation. Non-activated and TRAP-activated samples displayed an intense clot retraction by 60 minutes, while CLA-pretreated samples were much less retracted.

CLA effectively inhibits phosphatases in resting and activated platelets and can be used in a wide variety of platelet functional assays ranging from clot retraction, via calcium measurements, to thrombin generation. With the simultaneous use of selective platelet activators it can be utilized to dissect biochemical pathways during thrombus formation.

We investigated three monocytic leukemia cell lines, derived from a patient with de novo AML, a case of relapsed AML and from an AML case that developed secondary to myelodysplastic syndrome. We hypothesized that monocytic leukemic cells have a higher procoagulant activity thus, we characterized their thrombin generating potential by different assays using coagulation factor-deficient plasmas and TF neutralization. Results were compared to isolated normal human monocytes as negative- and to an acute promyelocytic leukemia cell line as positive controls. Because in previous studies microparticles from various cells have been shown to differentially modulate clot formation, we also tested the thrombin generating potential of the MPs derived from these leukemic cell lines.

In these experiments, we have found that monocytic leukemia cell lines and MP generated by these cells exhibit significantly higher PCA compared to normal monocytes and that this effect is primarily TF dependent; however, other factors may modulate this effect. Our results demonstrate that functional assays may provide a useful tool to identify cell induced thrombin generation in acute myeloid leukemias.

MAIN SCIENTIFIC RESULTS, OBSERVATIONS

1. After TRAP activation 25% of platelets showed PS- expression and cytosolic calcium transients during platelet function analysis.

2. TRAP activation in platelet rich plasma elicited faster thrombin generation and higher peak thrombin compared to non-activated sample.

3. The protein phosphatase inhibitor, CLA completely blocked the TRAP-induced PS-expression and thrombin generation in platelet rich plasma.

4. In CLA-pretreated samples clot retraction could not be observed.

5. By direct activation of intracellular IP3 receptors CLA could not blocked platelet activation. PP inhibition by CLA does not impair direct IP3 receptor stimulation, so its effect is exerted upstream of the IP3 receptor.

6. Acute monocytic leukemia cell lines have a significant procoagulant effect, that is primarily TF-dependent, FVII has an important role in it, while the propagation of thrombin generation is FXI dependent to a smaller extent FXII dependent.

7. Acute monocytic leukemia cell lines produce 4-10 microparticles in a 48-hour period and these microparticles are 100-fold more procoagulant than their parent cells.

8. The thrombin generation test that was set to examine the cell lines can be suitable to investigate the thrombin generation in clinical cases of acute myeloid leukemia.

KEYWORDS

Coagulation, thrombosis, platelet, calyculin-A, acute monocytic leukemia, tissue factor, microparticle, clot retraction, thrombin generation

LIST OF PUBLICATIONS

UNIVERSITY AND NATIONAL LIBRAY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/332/2017.PL
Subject: PhD Publikációs Lista

Candidate: Renáta Hudák
Neptun ID: UK710R
Doctoral School: Kálmán Laki Doctoral School

List of publications related to the dissertation

1. **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: 3.432 (2016)
2. **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/megjelenés alatt
   IF: 2.476 (2016)

UNIVERSITY AND NATIONAL LIBRAY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

List of other publications

3. **Hudák, R.**, Székely, E. G., Czuriga-Kovács, K. R., Nagy, A., Hofgárt, G., Berényi, E., Csiba, L., Kappelmayer, J., Bagoly, Z.: Low thrombin generation predicts poor prognosis in ischemic stroke patients after thrombolysis.
PLoS One. 12 (7), 1-13, 2017.
IF: 2.806 (2016)

4. Frendl, I., Katkó, M., Galgóczi, E., Boda, J., Zsíros, N., Németi, Z., Bereczky, Z., **Hudák, R.**, Kappelmayer, J., Erdei, A., Turchányi, B., Nagy, E.: Plasminogen Activator Inhibitor Type 1: a Possible Novel Biomarker of Late Pituitary Dysfunction after Mild Traumatic Brain Injury.
J. Neurotrauma. [Epub ahead of print], 2017.
IF: 5.19 (2016)

5. 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

6. Hevessy, Z., **Hudák, R.**, Kiss-Sziráki, V., Antal-Szalmás, P., Udvardy, M., Rejtő, L., Szerafin, L., Kappelmayer, J.: Laboratory evaluation of a flow cytometric BCR-ABL immunobead assay.
Clin. Chem. Lab. Med. 50 (4), 689-692, 2011.
DOI: http://dx.doi.org/10.1515/CCLM.2011.834
IF: 2.15

Total IF of journals (all publications): 18,343
Total IF of journals (publications related to the dissertation): 5,908

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

25 October, 2017

ACKNOWLEDGEMENTS

I would like to thank to my supervisor, János Kappelmayer his help and support in the previous 7 years from the beginning of my student research work till the end of my research. I would like to thank for the good research atmosphere useful advice and for the attitude that I could learn during my work in his Department.

I would like to thank Zsuzsa Bagoly who gave me very useful advice.

I would like to thank Péter Antal-Szalmás for consultations.

I would like to thank Ildikó Beke Debreceni and Gabriella Gál Szabó for the technical help during the experiments in the laboratories.

I am thankful for the help to the contributors working in the Hemostasis unit and Flow cytometry unit in Laboratory Medicine Department.

I would like to thank to Zsuzsanna Hevessy for the help during the immunophenotyping of the leukemia cell lines.

Thank to Csaba Antal, Ildikó Kópis, Levente Székely for administration work. I am thankful for the contributors working in Laboratory Medicine Department.

I am thankful for the support to my family and friends.