

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

**Coated-platelets. Subpopulation of hyperactive platelets.
Basic research and clinical observations.**

by Gyula Reményi M.D.

Supervisor: Udvardy Miklós D.Sc.



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VASCULAR DISEASES

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The Examination takes place at the Library of Department of Pediatrics, Medical and Health Science Center, University of Debrecen, at 11:00 a.m., 10 September, 2013

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The Ph.D. Defense takes place at the Lecture Hall of Building “A” of the Institute of Internal Medicine, Medical and Health Science Center, University of Debrecen, at 1:00 p.m., 10 September, 2013.

Introduction

Platelet stimulation with dual agonist collagen and thrombin leads to the formation of two populations of activated platelets, according to the surface α -granule accumulation. A subset of platelets, about 30 % presents 10-100 times more of these coagulant proteins. It was called COAT-platelets, an acronym for Collagen And Thrombin activated platelets. Convulxin (a GPVI agonist snake venom) is fully able to substitute for collagen in generation of these cells. The finding that additional agonist(s) can also produce a similar subpopulation of cells made the COAT-platelets changed to coated-platelets referring to the coating of adhesive and procoagulant proteins on the cell surface. This terminology focuses on the final cell product rather than the agonists used in its formation. Platelet stimulation other than convulxin + thrombin (thrombin + Fc receptor binding, high-dose thrombin, thrombin receptor agonist peptide (TRAP) or immobilized collagen) can also produce coated-platelets. The coated-platelet ratio upon dual agonist stimulation is influenced by temperature and unknown factors. At room temperature there are more coated-platelets produced than at 37 °C. Young platelets are prone to form more of these cells ($\approx 70\%$), than mature platelets. With the production of coated-platelets it has to be emphasized two things:

1. Upon dual agonist stimulation with thrombin and convulxin (0.5 U/mL thrombin and 500 ng/mL convulxin) **all** the platelets get activated.
2. During coated-platelet formation there are **two separate populations** evolving. One with certain amount of e.g. fibrinogen on the cell surface (about 70% of all platelets) and another one with 10-100 times more of the same protein (coated-platelets, about 30% of all platelets). There is no in-between state or a continuous spectrum.

Not just Factor V but other α -granule proteins are also presented on coated-platelets such as fibrinogen, von Willebrand factor (vWF), α_2 anti-plasmin (α_2 AP), fibronectin, thrombospondin. Alfa-granule proteins bound to coated-platelets are derivatized with serotonin. During transglutaminase reaction binding sites on the cell surface for serotonin-derivatized proteins are provided by fibrinogen and thrombospondin. The putative structure of the coated-platelet surface includes an intertwined network of α -granule proteins, each retained on the cell surface through multivalent interactions with membrane receptors and neighbouring proteins. Additional coated-platelet feature is the phosphatidylserine expression on the cell membrane. The coagulant protein coating (with high amount of Factor V) accompanied with negatively charged phosphatidylserine phospholipids results the third hallmark of coated-platelets: potent prothrombinase activity.

Main objectives

I. Basic research with coated-platelets

I/1. Connection between zinc ion and coated-platelets

Platelets contain high amount of zinc ion. Zinc plays important role in several biological processes and zinc deficiency causes bleeding tendency. There are data in the literature about the connection of low level of coated-platelets and diseases with bleedings. Using zinc chelators we studied the effect of zinc on coated-platelet formation.

I/2. Pore formation upon coated-platelet production

Using membrane impermeable dyes we made the new discovery that fluorescent dye was able to exit platelets upon coated-platelet formation. This phenomenon could explain by pore formation on coated-platelets. With different probe we tried to measure the size of

these pores and studied the effect of inhibition and activation of these pores on coated-platelet production.

I/3. Platelet-microparticles and coated-platelets

Platelet derived microparticles (P-MP) are the most common type of microparticles in human body. Their role of maintaining of normal haemostasis is well known. P-MP-s are also suspected to be involved under pathological conditions such as diabetes, hypertension etc. Flow cytometric detection of MP-s are cumbersome. We developed a new method to increase the sensitivity of flow cytometry. Using this technique we studied the connection of coated-platelet formation and MP production.

I/4. Role of CD9 tetraspanin in platelet-microparticles formation

We have shown that coated-platelet production accompanied with MP formation. Therefore we tested different agonist with coated-platelet production potential on their MP formation ability. Among others we used anti-CD9 antibody how it could affect MP production.

II. Coated-platelet research in essential thrombocythaemia (ET)

There is growing body of evidence of the role of coated-platelets in different diseases. It has been found increased coated-platelet levels in pathological conditions with thrombotic events (stroke, diabetes), on the other hand lower level of coated-platelets has been shown in bleeding tendencies (spontaneous cerebral bleeding, haemophilia A). It has not been studied yet in essential thrombocythaemia, condition with elevated platelet count. We compared coated-platelet levels of patients of our clinic to coated-platelet levels of healthy controls. We also evaluated the effect of hydroxyurea (HU) treatment on the lower than normal coated-platelet levels in ET.

Materials and methods

Preparation of gel-filtered human platelets

Five ml of whole blood was drawn from the antecubital vein into a plastic syringe containing 0.5 mL acid citrate dextrose buffer. After 1:2 dilution with buffered-saline-glucose-citrate (BSGC; pH 7.3) at room temperature (RT), platelet-rich plasma (PRP) was immediately prepared during centrifugation of the diluted samples in plastic tubes at 170 x g for 8 minutes at RT. Gel filtration was performed on a column of Sepharose CL-2B (Sigma-Aldrich, Saint Louis, MO, USA), and purified platelets were adjusted to the cell concentration of $4 \times 10^4/\mu\text{L}$ in BSGC.

Flow cytometric analyses

Subsequent platelet activation and immunostaining were assessed in a total volume of 100 μL containing Mix (79 μL), gel-filtered platelets with a final concentration of 40 G/L (10 μL), 1 μL biotinylated-fibrinogen (100 $\mu\text{g}/\text{mL}$) (Sigma-Aldrich), and agonists convulxin (1.25 $\mu\text{g}/\text{ml}$) (Pentapharm, Basel, Switzerland) and α -thrombin (5 U/ml) (bovine thrombin; Sigma-Aldrich) in 10 μl . Mix was prepared with the following components: 10 mM HEPES, 1 mg/mL BSA, 2.5 mM CaCl_2 , 1.25 mM MgCl_2 , and 150 mM NaCl. Different agonist combinations were also used: 5 U/mL thrombin + 10 μL A23187. Platelet labelling were additionally with 10 μL FITC-anti-fibrinogen antibody. For Bodipy staining immediately before use, 5 mM Bodipy-maleimide was diluted 1:10 in HEP/saline, and 5 μL of the diluted material was added to 1 mL of gel-filtered platelets for 5 min at room temperature; final concentration of Bodipy-maleimide was 2.5 μM . For activation/inhibition tests gel-filtered platelets were incubated with different materials e.g. 150 μM o-phenanthroline, 4 $\mu\text{mol}/\text{L}$ cyclosporin-

A, 10 µg/mL ALB6. In CD9 tests platelets were pretreated with 1 mg/ml (final) of IV.3 for 5 minutes at room temperature (RT) to block FcγRIIIa receptors and thereby prevent anti-CD9 mediated platelet activation. Anti-CD9 mAb at 10 mg/ml (final) were then pre-incubated with platelets for an additional 5 min at RT prior to activation with convulxin plus thrombin. Anti-αIIbβ3 mAb were tested similarly. Reaction was assessed for 10 minutes at 37°C, then was stopped with 200 µL 1% (wt/vol) paraformaldehyde in HEPES/saline and fixed for 20 minutes at RT. After fixation, 3.5 mL PBS containing 1 mg/mL BSA (PBS/BSA) was added, and the sample was centrifuged at 1500 x g for 15 minutes. The pellet was resuspended in 200 µL PBS/BSA, labelled with anti-CD41a-PE-Cy5 and streptavidin-PE (both from Becton Dickinson, San Jose CA) and centrifuged as above and finally resuspended in PBS. The levels of coated-platelets were determined in percentage (%). Detection of platelet P-selectin (CD62) percent positivity was performed as previously described. Basic research flowcytometric analyses were performed at the University of Oklahoma on a FACS-Calibur (Becton Dickinson, Mansfield, MA, USA) instrument with 15 mW argon laser and CellQuest software. Clinical research was performed at the University of Debrecen on BD FACS-Canto II instrument (Becton Dickinson, Mansfield, MA, USA), and data were analyzed via BD FACSDiva software (6.1.3 version).

Zinc measurement with fluorometry

To measure platelet zinc concentration Newport Green (Molecular Probes Europe) was used. The membrane permeable Newport Green acetate (NG-Ac) enters the platelet where the acetate part gets cut off by acetylase and becomes membrane impermeable. Gel-filtered resting or activated platelets were incubated in PBS with 25 µM NG-Ac for 30 minutes at 37 °C. After washing with PBS samples were analysed on

microwell plate by fluorophotometer(Fluorocount; Packard, Rungis, France) at 485 and 530 nm to PBS/25 μ M NG-AC blank.

b-BSA-(5-HT)₆ ELISA

Gel-filtered platelets were incubated in MIX with 10 μ g/mL b-BSA-(5-HT)₆ derivatized with azidotetrafluorobenzoic acid and activators at 37 °C as mentioned above. After 10 minutes at 37°C, the samples were placed on ice and cross-linked for 2 minutes at about 10 cm from a UV lamp (UV Crosslinker, FB-UVXL-1000; Fisher Scientific, Pittsburgh, PA). One μ g/mL chymotrypsin for 30 minutes at 37°C and cells were then lysed with 0.5% Triton X-100. Microtiter plates were coated with antibodies (10 μ g/mL) and blocked with 2 mg/mL BSA. One hundred μ L aliquots of platelet lysate were added to the wells for 90 min at RT. Bound b-BSA-(5-HT)₆ was detected with streptavidin-peroxidase.

Confocal microscopy

After activation, platelets and microparticles were labelled with Bodipy-maleimide, as detailed. Confocal images were acquired with a 488 argon, 568 nm Krypton, 633 nm Helium-Neon laser on a Leica TCS NT instrument (Heidelberg, Germany) with a 100x microscopic objective and 1.4 numerical aperture.

Animal experiment

BALBc mice were put on zinc free diet. Blood were drawn using ACD containing syringe by direct heart puncture and the mice were than sacrificed. Gel-filtered platelet rich plasma was used for coated-platelet assay as previously detailed.

Patients and methods in ET study

We measured the coated-platelet and P-selectin (CD62) levels by flow cytometry in 43 ET patients and 31 healthy controls. Patients were recruited from our clinical database of 175 ET patients. Diabetes and NSAID treatment were exclusion criteria. Fifteen of 43 patients took hydroxyurea (HU). Platelet functions were also tested by PFA-100 method.

Statistics

Data were compared with a Student's t-test, and statistical significance was set as $P < 0.05$. Experiments with MPTP modification of microparticle production were evaluated with a paired t-test. At ET patients' data Kolmogorov-Smirnov test was used for the evaluation of the normality of the data. Results were analyzed by using linear regression with adjustment for gender, reticulocyte percentage, and platelet count. Unadjusted between-group comparisons were done by analysis of variance (ANOVA) or Kruskal-Wallis test. $p < 0.05$ was regarded as statistically significant. Statistical analyses were performed by using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

I/1. Connection between zinc ion and coated-platelets

Introduction

Continuous high level of trace element of zinc is present in brain, heart, lungs and muscle tissue. Zinc content in blood, bone, testicles and hair are variable in proportion to the zinc uptake. Platelets have higher level of zinc (400-800 μM) compared to other cells and located mostly in cytoplasm and dense bodies of unknown reason. Zn^{++} ions are involved in almost every biological process from membrane stabilization to cell growth. Zinc deficiency accompanied with cutaneous bleeding tendency and impaired wound healing and growth, and neuropsychiatric disorders. Connections between zinc and primary haemostasis have been discussed in many papers. Zinc deficiency leads to bleeding tendency therefore zinc may have a role of coated-platelet synthesis.

Results

Zinc chelators have been tested on coated-platelet production. We recently observed that extracellular zinc chelators, o-phenanthroline, 5-amino-o-phenanthroline and TPEN, inhibited coated-platelet formation by dual (convulxin + thrombin) and triple (convulxin + thrombin + ADP) agonist stimulation; specifically, 150 μM o-phenanthroline attenuated coated-platelet production by $66 \pm 12\%$ and $40 \pm 17\%$ (mean \pm 1SD) respectively. A possible role for zinc in coated-platelet formation was demonstrated by the observation that μM zinc can potentiate binding of serotonin-derivatized α -granule proteins to immobilized fibrinogen. Specifically 50 μM zinc lowered the apparent EC_{50} for biotin-BSA-serotonin binding to fibrinogen by 56%. In addition, mice fed a zinc-deficient diet for 40 days showed a significant decrease in their ability to produce coated-platelets.

Discussion

We can say, that the exact role of zinc in platelet physiology is not clear yet. We demonstrated the surprising fact, that zinc chelators inhibit coated-platelet formation. During coated-platelet production platelets lost their zinc content by 40 %. Extruded zinc potentiates the build up of protein coating on platelet surface as facilitates the binding of serotonin-derivatized, α -granule proteins to serotonin binding sites on fibrinogen and thrombospondin. In our experiments zinc augmented the binding of serotonin-derivatized-albumin to fibrinogen in an ELISA format by dose dependent manner. Zinc deficiency causes bleeding disorder in animal model. We demonstrated that mice fed a zinc-deficient diet for 40 days showed a significant decline in coated-platelet production. Longer lasting diet decreased the coated-platelet percentage more.

I/2. Pore formation during coated-platelet production

Introduction

A surprising observation occurred with experiments designed to measure intracellular zinc levels using fluorescent zinc chelators. These data showed that the fluorescent chelator Newport green was lost from coated-platelets although platelet size did not altered significantly by flow cytometry. This observation was supported by the demonstration that intracellular calcein and fluorescein were also lost from coated-platelets. In addition, the two membrane-impermeable probes, FITC-phalloidin and biotin-maleimide, were incorporated into coated-platelets but other F-actin specific probe the FITC-DNase and FITC-anti-actin IgG did not, only in case of pre treatment with saponin, causing

membrane degradation. These lead to the hypothesis that a “pore” was formed in the membrane of coated-platelets.

Results

Several different size, membrane-impermeable probes were then utilized to demonstrate that this pore was capable of allowing molecules up to 2139 daltons, but smaller than 3380 daltons, to enter or exit the coated-platelet. This “pore” may also be at least one mechanism for extrusion of intracellular zinc from coated-platelets. Pore of this size exists temporary in the mitochondrial outer membrane during apoptosis, called mitochondrial permeability transition pore (MPTP). Apoptosis signal activates this pore, making the mitochondrion swell and lost of its potential causing cytochrome-C release into the cytoplasm and caspase-3 and caspase-9 activation. Flow cytometric analysis of coated-platelet production was used to examine the impact of pharmacological effectors of MPTP formation. Effects of MPTP inhibitors (cyclosporin-A, coenzyme Q, bongkreikic acid) and activators (atractyloside, phenylarsine oxid (PAO) and diamide) were detected by mitochondrial potential ($\Delta\Psi_m$) sensitive dye (JC-1) along with coated-platelet probes (fibrinogen retention, phalloidin uptake and phosphatidylserine expression). Phenylarsine oxide and diamide, both potentiators of MPTP formation, stimulate coated-platelet synthesis. Atractyloside, another inducer of MPTP formation, does not affect the percentage of coated platelets synthesized; however, it does increase the level of phosphatidylserine exposed on the surface of coated platelets.

Discussion

We can state that coated-platelet synthesis accompanied with membrane pore formation of size cut off of 2100-3400 dalton. An example of this pore is MPTP, playing a role in apoptosis. MPTP activation increases

and MPTP inhibition decreases coated-platelet formation. These findings indicate that MPTP formation is an integral event in the synthesis of coated platelets. Although the exact function of the MPTP remains to be determined, these data support a growing body of evidence that apoptosis-associated events are vital components of the platelet activation process.

I/3. Platelet-microparticles and coated-platelets

Introduction

Platelet microparticles are irregular small vesicles 0.1-1 μm of diameters released from activated platelets. These are the most abundant type of microparticles. Their main features are expression of proteins common with platelets' e.g. P-selectin, GPIb, GPIIb/IIIa, GPIV, GPV, GPIX, vWF, fibrinogen, FV, FVIII, thrombospondin, protein-S, caspase, cytochrom-C. Additionally they present negatively charged surface phosphatidylserine with potent prothrombinase and tenase activity enhancing coagulation. Pathological conditions with elevated numbers of platelet-microparticles are: SLE, sickle cell anaemia, paroxysmal nocturnal haemoglobinuria, chronic myeloproliferative disorders, diabetes, renal insufficiency and hypertension. Coated-platelets express surface phosphatidylserine as well, making a plausible connection between coated-platelets and platelet-microparticles. We used a cytoplasmatic fluorescent dye (bodipy-maleimid) with high fluorescent capacity for detection of microparticles.

Results

Confocal microscopy revealed, that platelets, preincubated with bodipy, upon dual agonist stimulation with convulxin and thrombin, produce high amount of microparticles of 0.3-0.5 μm of diameter. Microparticles generated by convulxin plus thrombin are positive for GPIIb/IIIa (using FITC-anti GPIIb/IIIa antibody). All microparticles are annexin-positive while only a fraction of the platelets are annexin-positive, i.e. phosphatidylserine expressing coated-platelets. Convulxin or thrombin alone makes a few microparticles in one hand, on the other hand calcium ionophore A23187 by itself or in combination with thrombin generates high amount of microparticles. Flow cytometry makes it possible to calculate microparticle-coated-platelet ratio with different agonist or agonist combinations (control, convulxin plus thrombin, convulxin, thrombin, Ca-ionophore plus thrombin, Ca-ionophore). Single agonist stimulation produces <1 microparticle/coated-platelets, not different from control's. However, using dual agonist or A23187 activation increases microparticle number. In average convulxin + thrombin or A23187 made 25% and 90% coated-platelets of all platelets respectively and microparticle/coated-platelet ratio was 12-20. These phenomenon was reproducible i.e. there is high correlation with coated-platelets and platelet-microparticles.

Discussion

Microparticles produced upon platelet activation may have an important role in haemostasis, especially in ITP, seeing that their negatively charged phosphatidylserine surface make an ideal place for prothombinase complex. The small size of microparticles makes it difficult to analyse them. New staining technique with bodipy-maleimide, developed by us, gives fluorescence in microparticle size

enough, that we can do quantitative measurement by flow cytometry. Different experiments with various agonists revealed strong correlation between coated-platelet formation and the number of platelet-microparticles produced.

I/4. Role of CD9 tetraspanin in platelet-microparticles formation

Introduction

CD9 glycoprotein (p24, motility related protein 1) is a member of the transmembrane 4 superfamily or tetraspanin (TM4SF). CD9 is present on platelets, white blood cells, endothelial cells, neurocytes, vascular smooth muscle, cardiac muscle, and epithelia. It has 4 transmembrane domains that make 2 extracellular loops and a short cytoplasmic C- and N termini. The tetraspanins have an ability to bind to each other or to other membrane proteins forming a big membrane molecular complex. CD9 is involved in many biological processes: cell growth, motility, cell adhesion, activation and signalling. The bound network of tetraspanin, the tetraspanin web, has an important role in cell fusion. To this it has to bind to surface integrins making a complex. An example for this kind of cell fusion is the sperm and oocyte fusion. CD9-deficient female mice showed sterility caused by a defect in the gamete fusion process. CD9 is an abundant platelet membrane protein approx. 45000 molecules/cell. The role of CD9 in platelet physiology is still obscured. Megakaryocytes need CD9 for their maturation. For GPIIb/IIIa activation CD9 binds to this integrin and to Fc γ RIIa. However, CD9 deficient mice do not have apparent haemostatic defect. We have shown an unexpected finding recently that an anti-CD9 mAB ALB6 plus thrombin produced average number of coated-platelet

without platelet microparticle formation. This discovery made us to think that CD9 is essential for platelet microparticle production.

Results

Four anti-CD9 antibodies were utilized to check coated-platelet production and microparticle production. The four antibodies (FMC, ALB6, SN4, ML13) were active against three different epitopes of CD9 and all of them inhibited significantly the microparticle production upon convulxin plus thrombin activation. This inhibition was more pronounced with platelet stimulation by A23187 plus thrombin. The negative effect of ALB6 lasted only at the beginning 2-3 minutes of the reaction and this checked up with the observation that coated-platelet formation happens within this period of time. Visualized by confocal microscopy we managed to show the absence of microparticles when ALB6 and convulxin plus thrombin were used as platelet agonists. It has been known that CD9 does not act in different biological processes alone but in tetraspanin web e.g. bound to integrins, therefore we investigated the effect of anti-GPIIb/IIIa monoclonal antibodies (AP2, Tab, abciximab) and small molecule GPIIb/IIIa inhibitors (eptifibatide, tirofiban and DMP-802) on microparticle production. AP2 antibody decreased the microparticle formation significantly ($p < 0.01$), other antibodies and inhibitors did not have the same effect however none of the molecules attenuated coated-platelet production.

Discussion

The role of CD9 in platelet physiology is not clear yet. Previous observations have shown that the anti-CD9 ALB6 attenuates microparticle production. This negative effect terminates after 2-3 minutes of reaction that makes another indirect connection between coated-platelet and microparticle production. The CD9 molecule has the

ability to bind to another CD9 molecule and other membrane proteins building up a tetraspanin web, responsible for biological effects. Four anti-CD9 monoclonal antibodies against three different epitopes of CD9 proved to work the same. These same effects on different epitopes are in favour of the fact that the antibodies sterically block the formation of tetraspanin web rather than block a specific CD9 and protein interaction. CD9 is in connection with GPIIb/IIIa therefore we tested GPIIb/IIIa inhibitors as well. AP2 an anti-GPIIb/IIIa monoclonal antibody significantly inhibited microparticle formation but other inhibitors did not. CD9 molecule and tetraspanin web have been known to work in cell fusion. This was the first observation of CD9 in cell degradation and microparticle production.

II. Coated-platelet research in essential thrombocythaemia (ET)

Introduction

Coated-platelets and their basic features have been discovered in the last decade. These hyperactive platelets may play an important role in different ischemic and thromboembolic diseases. Despite of these obvious assumptions clinical researches are still in germinal phase. However, there are already some results available: elevated coated-platelet levels compared to healthy controls have been found after coronary interventions, in cortical stroke, in transient ischemic attack (TIA), especially in hypertension. Additionally, recurrent non-lacunar stroke and interestingly Alzheimer disease have been proved to have higher-than-normal coated-platelet levels. Diabetes and chronic haemodialysis are also prone to possess elevated coated-platelet count. Contrarily, low coated-platelet levels have been found in spontaneous cerebral haemorrhages, in lacunar stroke and severe haemophilia A.

Animal models have shown that impaired haemostasis in Scott's syndrome may have been explained with low coated-platelet count.

We examined the role of coated-platelets in essential thrombocythaemia (ET). ET belongs to chronic myeloproliferative disorders. Its main feature is the sustain elevation of platelet count (>450 G/L) accompanied with thrombotic or haemorrhagic events. Fifty percent of ET cases has JAK-2 kinase V617F mutation. Thromboembolic complications are the main causes of mortality and morbidity. We can expect increased risk of both arterial (stroke, myocardial infarction, arterial occlusions) and venous (deep vein thrombosis, pulmonary embolism) thromboses. Haemorrhagic complications are less frequent and are typical with very high platelet count (>1500 G/L) and manifested by bruises and mucous membrane bleedings. The pathomechanism of abnormal platelet function with or without haemostatic conditions in ET is still under investigation, and seems to be contradictory at certain points. Previous observations have reported increased spontaneous aggregation, but, in turn, decreased epinephrine and collagen responsiveness during platelet aggregation have been also seen with a lower ristocetin cofactor/von Willebrand factor (vWF) antigen ratio, and reduced level of large vWF multimers. PFA closure times (CT) are usually prolonged in ET subjects regardless of having thrombotic/bleeding complications or not. Increased platelet and monocyte activation due to elevated levels of platelet-monocyte with platelet-neutrophil complexes and increased platelet P-selectin (CD62) positivity, higher number of immature platelets and exaggerated thrombin generation may also be responsible for thrombotic events especially in ET patients with JAK2-V617F mutation. In addition, acquired deficiency of antithrombin III, protein C, and protein S might contribute to thrombosis in ET as well. However, no ex vivo test

provided predictive information on the risk of thrombosis or bleeding in ET.

Results

There was a significant difference in age ($p < 0.006$), as ET patients were older than controls (61 ± 11.5 years vs. 49 ± 9.7 years of age). No difference was seen in gender (female/male: 32/11 vs. 24/7), and in the number of smokers and non-smokers (11 vs. 14 subjects). According to JAK2 mutation (V617F) status, 9 ET patients showed negative results, whereas 34 patients were JAK2 positive (79%). Coated-platelet levels were studied in 15 ET patients with no treatment along with 28 patients on hydroxyurea therapy. Platelet counts were significantly higher ($p < 0.0001$) in both non-treated and treated ET subgroups compared to controls (577 ± 161 G/L, 513 ± 127 G/L vs. 294 ± 67 G/L), but with no difference ($p = 0.2212$) between the two ET subgroups. Furthermore, within the platelet population L-PL count was significantly higher in both non-treated ($p = 0.0065$), and treated ET patients ($p = 0.0007$) (11.7 ± 8.2 G/L, 10.9 ± 6.0 G/L vs. 6.2 ± 3.0 G/L). However, MPV parameters were similar in all study groups (9.5 ± 0.6 fL, 9.0 ± 0.8 fL vs. 9.2 ± 0.8 fL). Significantly higher platelet P-selectin percentage was found in the ET group compared to healthy controls (CD62%: $1.64 \pm 1.16\%$ vs. $1.15 \pm 0.48\%$, $p = 0.0461$). There was no difference in CD62% between non-treated and treated ET patients ($1.66 \pm 1.56\%$ vs. $1.63 \pm 0.91\%$). These results were accompanied with prolonged PFA-100 CT values showing significant differences between ET individuals (regardless of being treated or not) and the healthy cohort with either cartridge (PFA coll/ADP: 98 ± 16 s, 102 ± 33 s vs. 88 ± 14 s; PFA coll/adr: 163 ± 55 s, 152 ± 26 s vs. 127 ± 27 s). In terms of coated-platelet percentage, non-treated ET patients showed significantly lower levels of

coated-platelets versus normal subjects ($23.1\pm 8.8\%$ vs. $37.6\pm 12.7\%$; $p=0.0008$). On the other hand, coated-platelet levels in the hydroxyurea-treated group ($34.1\pm 12.3\%$) were significantly higher ($p=0.0008$), which did not differ from the normal values ($p=0.3799$). The absolute number of coated-platelets was also calculated in each subgroup. We did not see an alteration in the coated-platelet number between non-treated ET patients and healthy subjects (129.1 ± 57.7 G/L vs. 115.2 ± 51.1 G/L; $p=0.403$). Moreover, there was no significant difference in coated-platelet count between non-treated and hydroxyurea-treated ET individuals (165.7 ± 78.8 G/L; $p=0.127$). We also analyzed the effect of JAK2-V617F mutation on coated platelet level. There was an interesting tendency that the lowest mean level of coated-platelets was seen in the non-treated JAK2-V617F positive patients (21.8%) in contrast to those without mutation and medication (30.1%). Furthermore, among individuals with JAK2 mutation, mean coated-platelet levels were more elevated at hydroxyurea treatment than in JAK2-negative patients (from 21.8% to 33.1% vs. from 30.1% to 36.6%).

Discussion

In recent years, a large number of publications described a special subpopulation of activated platelets called coated-platelets. Coated-platelets demonstrated higher or lower levels than normal in various diseases. Elevated coated-platelet formation has been implicated in DM, TIA, and coronary artery disease with a potential role in thrombotic complications. Larger amount of coated-platelets produced excessive quantity of platelet microparticles as well. On the contrary, severe bleeding conditions were also associated with the lower levels of coated-platelets in intracerebral haemorrhage, early haemorrhagic transformation in non-lacunar brain infarction, and in haemophilia A with frequent bleeding tendency. However, no data are available about

coated-platelet formation in haematological diseases with platelet abnormalities.

In our study, we first intended to analyze whether the levels of coated platelets were basically altered in ET regardless of any haemostatic abnormalities. Thus, we recruited ET patients with a negative history for bleeding or thrombotic complications. We postulated abnormal coated-platelet levels in ET compared to healthy conditions due to the pathogenesis of this myeloproliferative disease. ET usually occurs in elderly people that made difficult to find an age-matched healthy control group without underlying diseases or concomitant treatment. Therefore, significantly younger controls could be used for statistical comparisons, but other characteristics were matched to our ET patients. Due to the significant difference in age between the entire ET and control cohorts, we selected ET patients (n=6), who were age-matched to our normal subjects (52.2 ± 13.2 years vs. 49 ± 9.7 years, $p=0.322$). Platelet counts were significantly higher in the ET group, but we failed to show any difference in MPV. Although the number of large platelets was significantly increased in ET regardless of treatment, the ratio of L-PL was not high enough within the entire platelet population to affect the overall MPV values in our patient cohort. It is known that larger and thus younger platelets are usually more active. Similarly, in patients with immune-mediated thrombocytopenia with increased destruction of platelets, L-PL count was significantly elevated compared to those with aplastic anemia with platelet hypoproduction. Interestingly, reticulated platelet count and reticulocyte count were similar in all ET patients and controls. Impaired platelet function was observed via prolonged CT values in PFA-100 tests in our ET patients. Similarly, others also showed that such patients demonstrated longer CT results versus non-ET subjects. Simultaneously, CD62 levels were studied for detecting platelet reactivity. Elevated platelet P-selectin expression was detected

in our ET cohort versus healthy subjects as was demonstrated in previous studies. Novel and striking differences were seen in coated-platelet levels in ET. Non-treated patients showed significantly lower coated-platelet levels compared to our controls ($p=0.0008$). Among ET patients, the increase in coated-platelet percentage was not related to the increase in platelet count. On the other hand, the levels of coated-platelets were significantly higher in treated ET subjects ($p=0.0008$) approaching the values measured in healthy individuals, so hydroxyurea treatment seemed to have an effect on coated-platelet production in ET. We point out that these observations are based on patients without thrombotic or hemorrhagic complications, and hydroxyurea was the only treatment making a homogeneous study cohort for coated-platelet measurements. More importantly, we analyzed coated-platelet levels before and after hydroxyurea treatment in the same person. In three ET patients, hydroxyurea therapy significantly increased ($p<0.05$) the ratio of coated-platelets by 18% of average. To eliminate the potential bias of age on coated-platelet level, an age-matched ET subgroup ($n=6$) was compared to controls. We still found significantly lower coated-platelet percentage in these patients ($25.1\pm 5.2\%$, $p=0.018$) versus controls, which supported that coated-platelet level in ET was not an age-dependent parameter. In addition, we determined the absolute number of coated-platelets along with their percentage, but no such alteration in coated-platelet count was observed between ET and normal subjects what we detected in coated-platelet percentages. Previous studies reported only coated-platelet percentage in various diseases, thus, based on our results, we also suggest to analyze coated-platelet levels in percentage. Finally, the association between JAK2-V617F mutation and coated-platelet levels was studied. Based on our database that consists of 175 ET patients, 64.6% had JAK2-V617F mutation, which was a similar ratio to previously published data. However, in the present study group, this positivity was much higher being 79%. This alteration may

come from the strict patient enrolment and the relatively low number of patients. More importantly, the lowest mean coated-platelet ratio (21.8%) was seen in the non-treated JAK2-V617F positive patients. In contrast, mean coated-platelet percentage was similar in JAK2-positive subjects under hydroxyurea medication (33.1%), and all JAK2-negative individuals regardless of the medication (36.6%, 30.1%). It was previously described, that the presence of JAK2 mutation also interferes with other cellular activation events such as platelet P-selectin expression, and leukocyte-platelet interactions in ET. Based on former publications, coated-platelet production and apoptosis shared some common features, since both cellular events could be initiated by pro-apoptotic mediators such as BAX activators, and were inhibited by caspase inhibitors. Additionally, impaired apoptosis of megakaryocytes has recently been demonstrated in ET. Since hydroxyurea induces apoptosis in a dose-dependent manner, we propose that this drug increased apoptotic processes with an increased generation of coated-platelets regardless of the original platelet count. However, in JAK2-V617F positive subjects this elevation was somewhat less effective compared to the mutation negative patients. In ET patients, either thrombotic or hemorrhagic episodes may occur. Thromboembolic complications are usually well documented in medical records, but bleedings associated with ET may be unnoticed or often ignored. According to the WHO criteria, patients with high-risk of thrombosis are considered when being older than 60 years and/or have previous thrombotic event(s) in the history. Higher than 1500 G/L platelet count may be associated with high-risk of bleeding. In our study, no patient had either >1500 G/L platelet count or positive history because of the strict enrolment. Twenty-six patients were older than 60 years of age, but their coated-platelet levels did not differ significantly from younger ET patients ($28.9\pm 11.6\%$ vs. $32.6\pm 10.4\%$). We suggest that in ET the significantly lower levels of coated-platelets may contribute to platelet

dysfunction. Further follow-up studies are required to analyze the possible relationship between coated-platelet formation and the development of coagulation abnormalities.

New findings, observations

- Coated-platelet productions are influenced by both platelet and plasmatic zinc content. Zinc facilitates covalent bond of serotonin derivatized α -granulum proteins on the cell surface. Bleeding tendency observed in zinc deficiency may be the result of low coated-platelet ratio.
- The mechanisms of coated-platelet production and apoptosis are partially common e.g. mitochondrial permeability transition pore (MPTP). Inhibition or activation this pore increases or decreases coated-platelet formation respectively.
- Coated-platelets produce in average 15-25 platelet-microparticles/coated-platelet, important participants of haemostasis.
- CD9 tetraspanin is essential for platelet-microparticle production. Inhibition of CD9 blocks microparticle formation. CD9 is known to be involved in cell fusion. This is the first example of CD9 in the cell degradation.
- Coated-platelet levels in ET are significantly lower than in healthy controls, irrespectively of age and platelet count. Hydroxyurea, an apoptosis inhibitor, nearly restores this decreased coated-platelet level. JAK2 V617F mutation positive status accompanied with more attenuated coated-platelet production. Low coated-platelet level may contribute to thrombocytopeny in ET.

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Item Number:

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Candidate: Gyula Reményi

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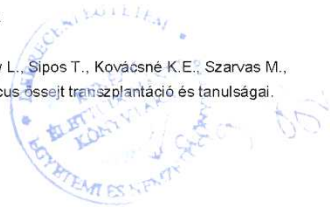
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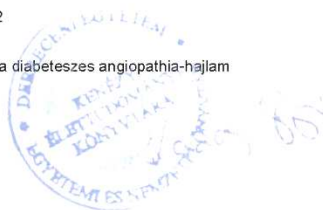


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