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PLATELET ACTIVATION MARKERS: IN VITRO EXPERIMENTS AND STUDIES IN PROTHROMBOTIC DISORDERS

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INTRODUCTION

Platelets are anucleated blood cells that originate from the cytoplasm of megakaryocytes in the bone marrow and circulate to maintain the integrity of the vascular system. In physiological conditions, this function contributes to hemostasis, the process that arrests blood loss after vessel injury. On the other hand, in diseases with several risk factors for atherosclerosis and atherothrombosis such as coronary artery disease (CAD), ischemic stroke or type 2 diabetes mellitus (DM), platelet function may lead to thrombotic occlusion of the vessel volume with obstruction of blood flow and subsequent tissue damage. Platelets are involved in both arterial and venous thromboembolism; however, they are more relevant to the process of thrombus formation in arteries. The main trigger for activation of platelets is the loss of endothelial cell barrier between extracellular matrix components and flowing blood resulting in the exposure of subendothelial collagen and von Willebrand factor (vWF). In addition, plaque rupture with tissue factor (TF) expression also induces platelet activation as a complication of atherosclerosis. The response of platelets to these events lead to three overlapping phases of platelet activation process called as initiation, extension and perpetuation. In the initiation stage, circulating platelets are captured and then activated by exposed collagen and vWF causing the adhesion of a monolayer of platelets. Thrombin is generated on cell surface with high levels of phosphatidylserine (PS) after exposing to TF from vessel wall initiating the coagulation cascade and later platelet aggregation. Integrin receptors on the platelet surface are key components of these early events as integrin $\alpha_2\beta_1$ and glycoprotein (GP) VI bind collagen directly, while GPIbα and GPIIb/IIIa ($\alpha_{IIb}\beta_3$) receptors bind vWF and thus collagen resulting in intracellular signal transduction and an increase of intracellular Ca^{2+}. In diseases with thrombosis and inflammation, platelets may also be primarily activated by thrombin via protease-activated receptors (PAR1 and PAR4) of G protein-coupled receptors (GPCRs). During the extension phase, additional platelets are recruited, activated and stick to each other on the platelet monolayer, when adenosine diphosphate (ADP) is secreted from dense granules, and α-granule contents like P-selectin is expressed with many other biomarkers. The surface level of exposed P-selectin increases rapidly in order to mediate different cell-cell heterotypic interactions between platelets and leukocytes or endothelial cells. Simultaneously, thromboxane A_2 (TXA_2) is *de novo*

generated from arachidonic acid and then also released. ADP and TXA_2 along with accumulated thrombin activate platelets through GPCRs-mediated pathways. These events support more secretion, and the GPIIb/IIIa receptor activation as a result of inside-out signaling, and thus fibrinogen binding to platelets that is essential in full platelet aggregation and stable plug formation. PS- and TF-positive microparticles (MPs) with the size of 0.1-1 μm are shedded from activated platelets by a budding process to facilitate coagulation. Perpetuation is the late stage of platelet activation, when the platelet plug is stabilized by close contacts between platelets via outside-in signaling induced by receptor-bound ligands. These junctions thus prevent the dissolution of plug by supporting the accumulation of secreted agonists and shedded adhesive receptors like soluble P-selectin in the gaps between platelets. Finally, clot retraction occurs to narrow the interplatelet spaces resulting in a smaller, shrunk volume of stable plug.

Accordingly, activated platelets are the key components in many vascular disorders with arterial thrombosis: in the different forms of CAD such as myocardial infarction (MI) or stable/unstable angina, in addition to ischemic stroke, and peripheral artery disease (PAD). Moreover, in numerous other states like obesity, type 2 DM, and after invasive cardiological intervention (stent implantation) in patients with CAD, activated platelets are present in the circulation resulting in further thrombotic and inflammatory complications. The fast and efficient investigation of platelet activation markers - by using different technical approaches - is essential in the detection and follow-up of the short-term and long-term consequences of increased platelet activation. Moreover, it is also useful to monitor the effect of different antiplatelet regimens in patients as a screening test.

In the last two decades, the surface expression of P-selectin receptor has been considered as one of the most sensitive, “gold standard” activation-dependent events investigated by flow cytometry. Its analysis can be readily used for routine diagnostic purpose in addition to the soluble form of P-selectin after shedding into the plasma measured by enzyme-linked immunoassay (ELISA). Along with alternative activation markers like high MP levels as well as elevated amount of platelet-leukocyte heterotypic aggregates, all these data can be applied for a comprehensive analysis of platelet activation status in *ex vivo* clinical samples. Furthermore, investigation of *in vitro* stimulated platelets by platelet agonists may help to introduce the testing of additional activation markers and provide new insights into the mechanism of these complex events.

THE AIM OF THE DISSERTATION

Our aim was to investigate the levels of distinct markers of increased platelet activation in *ex vivo* studies from patients who suffered from different clinical diseases with high risk for prothrombotic complications, and also in stimulated platelets by *in vitro* experiments.

For this purpose we decided

1. to measure the levels of platelet and soluble P-selectin in obese and type 2 DM patients versus healthy subjects, and to establish the effect of the most intensively examined variant of P-selectin gene polymorphism (Thr715Pro) on soluble P-selectin levels in these patients and controls,

2. to investigate the direct platelet-activating effect of stent implantation via studying the levels of platelet-derived MPs (PMPs) and other platelet activation markers (platelet and soluble P-selectin, platelet-leukocyte heterotypic aggregates) at an early (15 minutes) time point after stenting with bare metal stent in patients with CAD, and these results were compared to data obtained from subjects with diagnostic catheterization alone,

3. to study *in vitro* the binding of blood coagulation factor XIII (FXIII) to activated platelets by measuring the FXIII-A surface positivity of whole blood and washed human platelets stimulated with thrombin-receptor activating peptide (TRAP) in healthy subjects and a type I Glanzmann thrombasthenia (GT) patient as a control, and analyzed these samples by flow cytometry,

4. to observe whether the non-active FXIII-A_2B_2 binds directly (to GPIIb/IIIa receptors) on stimulated platelets, or this binding is mediated via GPIIb/IIIa receptor-bound fibrinogen with the γ'- or γA-chains.

PREVIOUS STUDIES

Platelets play a key role in the development of atherosclerosis and related thrombotic complications in CAD, DM and several other diseases. The initial step for the development of atherosclerosis is associated with the dysfunction of endothelial cells as a result of the deposition of lipids in the vessel wall and the increased oxidative stress. These early events result in inhibition of nitric oxide production, increased adhesiveness of endothelial cells for platelets and leukocytes due to the higher prothrombotic activity and secretion of proinflammatory molecules. Platelets become hyperactive induced by the metabolic alterations (hypercholesterolemia, hyperglycemia), and the increased levels of adhesive molecules (ICAM-1, endothelin-1) and cytokines (interleukin-1β, tumor necrosis factor-α) released from endothelial cells and leukocytes. Different interactions between platelets and endothelial cells generate signals to recruit inflammatory cells to the site of vascular injury and promote the extravasation of more leukocytes to the inner layers of the vessel wall. Adhesion of activated platelets to atherosclerotic lesion alters plaque progression and may induce prothrombotic conditions by the release of further adhesive proteins like P-selectin. Binding of several plasma molecules (e.g. fibrinogen) on the platelet surface, generation of PS and TF-positive PMPs, and secretion of cytokines, such as CD40L and RANTES (regulated on activation, normal T cell expressed and secreted) occur simultaneously. Accordingly, platelets have a central role in the propagation of these inflammatory and prothrombotic events via several cell-cell interactions, which is mediated typically through the association of P-selectin with its counter-receptors on different cell types. The significance of the distinct roles of P-selectin in hemostasis is even more evident from the studies in P-selectin-deficient mice, which demonstrated a number of defects in leukocyte function (adhesion, rolling, recruitment), and platelet function caused prolonged bleeding time, and smaller and instable thrombus formation.

The P-selectin receptor (Granule Membrane Protein, [GMP-140]; Platelet Activation-Dependent External Granule Membrane, [PADGEM]) is a 140-kDa integral membrane glycoprotein located in the α-granules of platelets and the Weibel-Palade bodies of endothelial cells. This receptor is one of the members of the selectin family, which contains P-, E-, L-selectin that bind to a dimeric mucin (P-selectin glycoprotein ligand-1, [PSGL-1]) expressed on the surface of leukocytes and platelets. Selectins consist

of a lectin domain followed by an epidermal growth factor (EGF)-like module, varying number of short consensus repeat, a transmembrane domain and a short cytoplasmic tail. E-selectin is present only on activated endothelial cells, while L-selectin in constitutively expressed on the leukocyte surface.

P-selectin-PSGL-1 interaction is involved in leukocyte adhesion to endothelial cells and platelets, when P-selectin is expressed on the cell surface upon cell activation. Flow cytometry is the most advantageous technique for the analysis of platelet receptors especially for P-selectin exposure. Whole blood flow cytometric assay for platelet activation using a fixed specimen has been increasingly used over recent years to assess platelet function in clinical research settings. This approach offers several advantages over other clinical tests of platelet function including relative simplicity, minimal manipulation of samples, preventing artificial *in vitro* platelet activation, analysis in the physiological milieu of whole blood, high sensitivity for detection of platelet subpopulations, and the need for only minimal blood volumes. The preanalytical conditions are extremely important in the analysis of platelet activation markers. In addition, rapid fixation of blood after venepuncture is also critical. We found that the preparation of platelet-rich plasma (PRP), or even washed platelets resulted in a "false" up-regulation of P-selectin resulting in higher control values compared to whole blood analyses. Most authors in different *ex vivo* clinical studies reported less than 1-2% positivity for platelet P-selectin in whole blood in healthy subjects. Values above this rate means an enhanced platelet activation status and may predict a hypercoagulable state. According to our *in vitro* studies, platelets showed increasing level of P-selectin up to 90% in agonist concentration-dependent manner. A theoretical disadvantage of flow cytometry relates to the possibility that activated platelets may display a decreased survival in circulation, resulting in modest or non-evident platelet activation by flow cytometric analysis. However, convincing experimental data support that activated platelets continue to circulate. Thus, platelet activation is not necessarily the ultimate stage of platelet life.

The soluble form of this receptor has been identified in plasma as an alternatively spliced molecule without the transmembrane domain released by the cleavage of the membrane-bound form from the cell surface. In normal plasma, soluble P-selectin concentration showed difference between genders being higher in males. It seems that the primary contributors to the formation of soluble P-selectin in physiological state are platelets, and thus baseline concentrations are mostly determined by platelet P-selectin

shedding. The positive correlation between platelet count and the level of soluble P-selectin also supports this conclusion. In patients with bone-marrow aplasia, the soluble P-selectin concentration was reduced, but was not completely absent. The remaining soluble P-selectin in bone marrow aplasia could be derived from a few persisting megakaryocytes and/or platelets or from endothelial cells. Therefore, a minimal release of that from endothelial cells in normal situation cannot be excluded. Others found that progression of vascular, endothelial damage in essential, renovascular and malignant hypertension was associated with a rise in circulating levels of P-selectin, which also confirmed the link between soluble P-selectin and endothelial cells as a source of this protein. The shedding mechanism of P-selectin has not been exactly elucidated. Though no direct proofs have been reported, some metalloproteinases might be candidates causing P-selectin shedding as has been found for L-selectin.

Significantly elevated levels of platelet and soluble P-selectin have been found in a number of different diseases like unstable angina, ischemic stroke, PAD, atrial fibrillation, or DM compared to healthy control subjects. Moreover, elevated soluble P-selectin might serve as a predictive marker for future cardiovascular events, such as MI and stroke.

The P-selectin gene is located on chromosome 1q21 to 1q24 and it is highly polymorphic. Among the 13 gene missense polymorphisms, the Thr715Pro variant has been the most intensively studied, while it may have the most considerable effect of the function of P-selectin. It is located in the last consensus repeat region of the P-selectin molecule, and it is possible that the substitution of threonine for proline may induce a conformational change in the precursor protein, which may influence its intracellular transportation and secretion. Contradictory data have been published about the association of Thr715Pro polymorphism with cardiovascular disease. The presence of Pro715 allele has been shown to have a “protective” effect on MI in two extensive studies, but some authors reported no such effect. Anyway, Thr715Pro genotype seemed to have a substantial effect on the soluble P-selectin levels, but it is still unknown whether the Pro715 allele is associated with the reduction in the membrane bound form of P-selectin, or shedding of distal fragments of the receptor. Other polymorphisms (e.g. Ser290Asn, Asn562Asp) of this gene carried by the same haplotype were associated with an increased risk for MI in a study investigating French and Northern Irish populations. On the other hand, the Thr715Pro phenotype together with Ser290Asn and Asn562Asp polymorphisms were

excluded to be as major contributors to macrovascular complications in type 2 DM. Moreover, several additional polymorphisms have also been described in the promoter region of the P-selectin gene, no significant associations of -1817 T/C, -1969 G/A, and -2123 C/G polymorphisms with the soluble P-selectin levels were seen. Nevertheless, the exact association between the presence of mutated alleles or specific haplotypes of P-selectin gene and their impact related to soluble P-selectin levels in several diseases, was still unclear.

Degranulated platelets mostly aggregate with monocytes and neutrophils via P-selectin and PSGL-1 interaction. The levels of these heterotypic aggregates can be investigated by flow cytometry when platelet-specific antigens (e.g. CD41a [GPIIb] or CD42a [GPIX] positivity) are analyzed in the subpopulations of leukocytes to measure indirectly their interactions with platelets. The presence of these cell-cell aggregates has been shown to be a superior indirect marker of platelet activation in experiments *in vitro* and in *ex vivo* patient samples. The half-life of detectable circulating monocyte-platelet aggregates was much longer (cc. 30 min) than that of P-selectin-positive platelets (cc. 5 min) because of the rapid loss of P-selectin expression *in vivo*. However, in our preliminary *in vitro* studies, platelet P-selectin sensitively reflected the status of platelet activation compared to other platelet markers at low thrombin concentrations. Anyway, the analysis of platelet activation markers at an early time point is necessary to detect elevated activation of platelets effectively.

MPs are mostly derived from activated platelets, since PMPs constitute the largest subpopulation of all MPs. When MPs are isolated from normal human plasma, most MPs express platelet-specific markers (e.g. GPIX), and also P-selectin as being activated. *In vitro*, platelets were fragmented at high thrombin concentrations (0.5 U/ml) that were easily detectable by forward scatter (FSC) and side scatter (SSC) analysis. P-selectin expression increased gradually by elevating concentration of thrombin, while significant increase of the event count in the MP gate was only evident at high thrombin stimulation. However, these vesicles may also be released from endothelial cells, erythrocytes, leukocytes, and even megakaryocytes. MPs are procoagulant because of high level of PS with almost 3 times higher density than on activated platelets, to where Annexin V protein binds specifically in the presence of Ca^{2+}. In addition, a smaller portion of MPs is highly procoagulant by expressing not only PS, but also TF. P-selectin on activated platelets and endothelial cells and shedded soluble P-selectin bind to PSGL-1 receptors on monocytes

and this event induces TF-positive MP generation. Furthermore, P-selectin on platelets in thrombi promotes the recruitment of these MPs into the thrombus by binding to PSGL-1 on the MPs. This leads to increased thrombin generation at the site of injury.

Especially in the last decade, the pathological role of elevated PMP levels as a long-term consequence of platelet activation has been investigated in several diseases such as in the different forms of CAD, hypertension, paroxysmal nocturnal hemoglobinuria, chronic renal failure or in cancer. At least five different variables may contribute to the results obtained in normal and pathological samples. The gauge of needle, the type of anticoagulant, type of sample (MP suspension or MPs in platelet-poor plasma [PPP]), conditions for centrifugation and storage conditions. The source of sample (intracardiac catheter or peripheral blood) may also affect the results, however only a few studies investigated blood samples drawn directly from the stenting area. Thus, the total number of PMPs varies widely among different reports. Some former studies measured much higher mean PMP levels in PPP with larger variability (1137 ± 790 /µl plasma) compared to those, which analyzed PMPs in MP suspension (262 ± 146 /µl plasma). Cellular contamination may contribute to the very high PMP levels in PPP samples, hence, we also used MP suspension in our experiments.

Percutaneous coronary intervention (PCI) with coronary stenting is a widely administered invasive cardiological procedure in acute and elective cases of CAD with occluded or stenotic arteries in patients with stable or unstable angina or myocardial infarction. This invasive intervention may result in endothelial injury and inflammation resulting in high levels of activated platelets with significantly increased levels of PMPs. Such complex events may cause subacute and sometimes late stent thrombosis when platelets and PMPs adhere to and thus occlude to the stent area. This is one of the major limitations of invasive cardiological interventions with the possibility of life-threatening complication. Thus, the measurement of PMP level at a very early time point of sampling could make it possible to gain a new insight into the direct platelet-activating effect of stenting in these conditions.

As described above, platelets contain several biomarkers in their granules and release them upon activation such as P-selectin, thromboglobulin, and thrombospondin from α-granules, and lysosome-associated membrane protein [CD63] from lysosomes. In addition, other types can be shown in the cytosol like CD40L, which is also expressed by activated platelets after being secreted. Coagulation FXIII is also found in platelet

cytoplasm in large quantity, but its faith and role are not fully understood. FXIII is a protransglutaminase that is essential for maintaining hemostasis as a key regulator of fibrinolysis. Plasma FXIII is a heterotetrameric zymogen (FXIII-A_2B_2) that consists of two potentially active A subunits (FXIII-A) and two carrier/inhibitory B subunits (FXIII-B). The cellular form of FXIII (FXIII-A_2) that is a dimer of two A subunits, is present in monocytes, macrophages and the platelet cytosol. Platelet-bound FXIII is targeted and concentrated at the site where platelet-rich thrombi are formed. FXIII increases the fibrinolytic resistance of platelet-rich thrombi through the cross-linking of α_2-antiplasmin to fibrin and the presence of platelets accelerates the cross-linking process. The interaction between activated FXIII (FXIIIa) and platelets has been investigated in a few former studies, and a saturable and specific binding of FXIIIa to thrombin-stimulated, but not to resting platelets was demonstrated. However, data on the binding site of FXIIIa were contradictory. In the first study, GPIIb/IIIa receptor and platelet-bound fibrin(ogen) were ruled out as binding site for FXIIIa; RGD peptide did not influence the interaction between FXIIIa and platelets, and FXIIIa binding to platelets from two patients with severe Glanzmann thrombasthenia (GT) was normal. The binding of FXIIIa to thrombin-activated platelets was inhibited by plasmin. In a later study, however, the GPIIb/IIIa receptor was shown to mediate the binding of FXIIIa to thrombin-stimulated platelets, and indirect binding of FXIIIa through fibrinogen associated with its receptor was also demonstrated. However, it was not studied whether FXIII in non-activated form also can bind to platelets directly or via γA/γ' and γA/γA fibrinogens, and then on the surface of activated platelets it becomes easily activated by locally formed thrombin and exerts its cross-linking action.

SUBJECTS AND MATERIALS

Obese, type 2 DM patients and healthy controls

In the study to investigate the effect of the Thr715Pro P-selectin polymorphism on soluble P-selectin levels, we recruited 119 type 2 DM patients (diagnosed according to WHO criteria), 57 healthy volunteers with body mass index (BMI) <25 kg/m^2, and a BMI-matched non-diabetic study group consisting of overweight and obese subjects (n=48). Patients were enrolled from the Outpatients Clinic of the 1st Department of Internal Medicine, University of Debrecen. Type 2 DM patients were treated as required by antihypertensive, oral hypoglycaemic agents or diet, and none of them had thromboembolism. Other exclusion criteria were severe symptomatic vascular diseases such as angina, intermittent claudication, transient ischemic attack, malignancy, pregnancy, impaired liver or renal function and infectious diseases. Healthy controls did not suffer from cardiovascular, neoplastic, metabolic or inflammatory disease, as observed by careful examination and routine laboratory tests. Overweight and obese subjects were without history or clinical evidence of diabetes, however 6 individuals displayed impaired glucose tolerance and 15 subjects had mild hyperlipoproteinemia. An additional classification of type 2 DM patients was also made, that allowed a pairwise comparison of a subgroup of diabetic patients (age-matched DM group, n=57) with healthy controls.

Patients with stable angina underwent stenting or catheterization

We studied the direct effect of PCI on platelet activation via measuring the level of PMPs. 25 patients (20 men and 5 women; mean age: 61.69±12.65 years) with stable angina were recruited, who underwent stenting with non-drug eluting bare metal stent in the Department of Cardiology, University of Debrecen. Results were compared to 20 age-matched control individuals (15 men and 5 women; mean age: 59.6±6.65 years) suffered from stable angina, but with no eligible coronary stenosis for stent implantation, who underwent diagnostic coronary catheterization only, and were not stented. All participants were on aspirin (100 mg/day) monotherapy prior to the intervention, with no other antiplatelet medications. All individuals avoided taking other drugs that might have affected platelet function for 7 days before sampling. No significant difference in baseline laboratory parameters was found between the two study groups. Diagnostic catheterization was carried out in all patients and stenting was processed in case of significantly stenotic

or occluded coronary artery was found. During the intervention an intravenous bolus of 100 IU/kg Na-heparin were administered to all patients. Since there was no difference in the medication in the individuals of both groups, we could readily compare the early platelet-activating effect of the two distinct invasive procedures in the same disease state. No bleeding or thrombotic complications were recognized during and after the procedures for 30 days. Investigated subjects gave informed consent to the study, which was in accordance to the Declaration of Helsinki.

A patient with Glanzmann thrombasthenia

We analyzed the binding of non-activated FXIII to whole blood and washed platelets stimulated with TRAP in healthy individuals. In order to test the role of GPIIb/IIIa receptor in FXIII binding, platelets with a severe type I GT (GPIIb<200 receptors) were obtained from an 8-year-old patient treated at the Department of Pediatrics, University of Debrecen.

Reagents

The following reagents were used during the *in vitro* experiments: apyrase, prostaglandin E_1 (PGE_1), bovine serum albumin (BSA), TRAP, purified fibrinogen from human plasma containing FXIII (6.2 μg/mg fibrinogen), RGDS (Arg-Gly-Asp-Ser) tetrapeptide, paraformaldehyde (PFA), eptifibatide (Integrilin®), FITC-labeled monoclonal mouse anti-human-FXIII-A antibody, PE-labeled anti-human-P-selectin (CD62) antibody, non-immune mouse IgG_1 (isotype control) antibody, PerCP-conjugated monoclonal antibody to GPIX (CD42a) antibody and highly purified human FXIII-A_2B_2 prepared from the plasma of healthy volunteers. Human plasma fibrinogen variants γA/γA and γA/γ' (peak 1 and peak 2) were distinguished by DEAE-cellulose gradient elution chromatography. Peak 1 and peak 2 fibrinogens were essentially FXIII free (FXIII content was less than 0.1 μg/mg fibrinogen). FXIII content of all fibrinogen variants was measured by one-step sandwich ELISA, which is specific for the tetrameric plasma FXIII. The monospecificity of anti-FXIII-A antibody was tested by intracellular staining of FXIII content in permeabilized platelets. All other substances were of reagent grade.

METHODS

Blood drawing and sample preparation

Venous blood from patients and their controls was obtained into Vacutainer tubes containing 0.105 M sodium citrate by atraumatic venepuncture. Blood sampling conditions were designed to avoid artefactual activation of platelets during phlebotomy. In whole blood experiments, - within 2 hours of collection - 40 µl of patient and control samples were fixed in 1 ml 1% PFA and kept at room temperature (RT) for minimum 1 hour. Platelet number was determined in each case by Advia 120 Hematology System. Fixed whole blood samples were centrifuged at 1300 x *g* for 15 minutes at RT. The pellet was washed in 1 ml phosphate-buffered saline (PBS) buffer, then centrifuged as above and finally resuspended in PBS.

In order to study the amount of PMPs, PPP was obtained from whole blood anticoagulated with sodium citrate by centrifugation at 1550 x *g* for 20 minutes at RT. Five hundred µl of PPP was spun down at 13000 x *g* for 2 minutes to get rid of platelet debris, and then centrifuged at 16100 x *g* for 30 minutes at RT to isolate MPs. We used this protocol with some minor modifications.

In the *in vitro* experiments, blood was dropped into Vacutainer tube containing ACD (38 mM citric acid, 75 mM sodium-citrate, 136 mM glucose) and 300 ng/ml PGE_1 was added before any further procedure to achieve minimal platelet activation during up-coming sample manipulation. Platelet–rich plasma (PRP) was prepared by centrifugation at 150 x *g* for 15 minutes at 37°C and then platelets were sedimented by centrifugation at 1200 x *g* for 15 minutes at 37°C. The platelet pellet was washed three times in a modified HEPES-Tyrode's buffer (140 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.1% glucose, 0.36% BSA, 10 mM HEPES, pH 7.4) in the presence of 1 U/ml apyrase and 300 ng/ml PGE_1. After washing platelets were finally resuspended in the HEPES-Tyrode's buffer without apyrase and PGE_1.

Flow cytometric analysis of clinical samples

Platelets were identified by a FITC-conjugated monoclonal antibody to GPIX (CD42a). Platelet activation was detected by PE-labeled anti-P-selectin antibody. Fixed platelets were incubated with saturating concentrations of antibodies for 20 minutes in the

dark at RT. As a control for immunolabeling with anti-CD62, platelets were incubated with PE-coupled non-immune mouse IgG_1 antibody and 10 000 dual-color labeled platelet events were acquired on a FACSCalibur flow cytometer by using the CellQuest 3.2 software.

The levels of platelet-leukocyte aggregates were investigated from whole blood when CD42a positivity was analyzed in the different subpopulations of leukocytes to measure indirectly their interactions with platelets.

In the MP study, we used and compared two sets of fluorescent beads TruCOUNT® and CytoCount® with standard size and amount for enumeration of MPs. Beads were not added directly into samples during the analysis, since in our preliminary experiments we had found a decrease in MP number due to the possible attachment of MPs to some beads. Thus, beads were first processed, and then clinical samples were measured within a standard collection time (30 seconds). The numbers of MPs were calculated based on the event count from the bead tube collected for the same time period. MP preparation was processed at exactly 120 minutes after sampling when separation was feasible in each patient. MPs were gated into a restricted area by FSC and SSC parameters, and then identified by their Annexin V and CD42a positivities. Furthermore, anti-CD62 antibody was also used to measure the P-selectin expression on MP surface as the marker of their activation status.

Laboratory assays

Soluble P-selectin levels in plasma were analyzed by ELISA commercial kits following the manufacturer's instructions. All plasma samples were centrifuged immediately at 2000 x *g* for 15 minutes at RT, aspirated and stored at -70°C until analysis. This procedure was completed within 30 minutes of blood drawing.

Blood glucose, total cholesterol, and triglyceride values in patients and their controls were measured on Hitachi analyzer and LDL-cholesterol levels were calculated by the Friedewald formula. HbA1c was measured by HPLC and the fibrinogen levels were determined by the Clauss-method. CRP was measured by a turbidimetric assay.

Genetic analysis of Thr715Pro P-selectin polymorphism

Genomic DNA was extracted from whole blood anticoagulated with sodium citrate by QIAamp DNA blood kit. Primers were designed by Primer3 and used to amplify exon 13 of P-selectin gene. The sequences of the oligonucleotide primers were 5′-TTTCTGCAGCTGTGAAATGC-3′ and 5′-ATTGTACCTTGGCAGGTTGG-3′. Polymerase chain reaction (PCR) was performed in a total volume of 50 µl containing 100 ng of DNA, 10 pmol of each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 10% DMSO and 2 units Taq DNA polymerase. After the initial denaturation at 94°C for 5 minutes, amplification was carried out for 40 cycles of 94°C for 30 seconds, 60°C for 60 seconds and 72°C for 60 seconds, and the final extension at 72°C for 10 minutes. In restriction fragment length polymorphism (RFLP), the PCR product (198 bp) was digested by Eco91I and the digested products were run on a 3% agarose gel and visualized under UV light by ethidium bromide staining. In the presence of Thr715Pro mutation, a new (163 bp) DNA product could be detected during analysis.

Analysis of FXIII binding to stimulated platelets by TRAP

First, FXIII binding to platelets was observed in whole blood. Citrated whole blood was stimulated with TRAP (0-40 µM) for 15 minutes at 37°C in HEPES-Tyrode's buffer containing 5 µl FITC-labeled anti-human-FXIII-A antibody (1 µg/ml). Platelets were identified by their CD42a positivity. Platelet activation was detected using anti-P-selectin (CD62) antibody in a three-color-labeling during flow cytometric analysis. As a control for immunolabeling, platelets were incubated with non-immune mouse IgG_1 antibody. After activation, samples were processed as described in "Blood drawing and sample preparation".

To investigate the origin of FXIII bound to stimulated platelets in whole blood, washed human platelets were also stimulated and labeled under the same condition as above. Results were expressed as percentage of FXIII-A positivity of platelets. In order to evaluate the FXIII-A and CD62 positivity on platelets with different size, the whole platelet population was divided into three equal-sized subgroups according to their FSC signal. Large, medium and small platelets were individually analyzed at the same cut-off of fluorescence intensity.

Measuring the effect of GPIIb/IIIa antagonists on FXIII binding

To test the role of the GPIIb/IIIa receptor in FXIII binding, we used two pharmacological approaches. GPIIb/IIIa receptor antagonist eptifibatide (Integrilin®) was used as an agent with high specificity that blocks the final common pathway in platelet aggregation by inhibiting the binding of fibrinogen to the receptor on the surface of activated platelets, which then decreases cross-linking of platelets and thus platelet aggregation. Furthermore, the effect of tetrapeptide containing RGD-sequence (RGDS) was also examined, which interferes directly with the interaction of fibrinogen and its platelet receptor. Thus, citrated whole blood samples were preincubated with eptifibatide (2 μg/ml) or RGDS peptide (5 mM) for 15 minutes at 37°C. After preincubation, platelets were stimulated with different concentrations of TRAP and studied by flow cytometry under the same conditions as described earlier.

Studying FXIII binding in Glanzmann thrombasthenia

We confirmed the involvement of GPIIb/IIIa receptor in FXIII binding in further experiments using whole blood sample from a patient with severe type I GT with very low level of platelet GPIIb/IIIa receptors. Citrated whole blood was stimulated with increasing concentrations of TRAP for 15 minutes at RT. Results from this sample were compared to the platelets of healthy controls according to the FXIII-A % positivity.

Analysis of the mechanism of FXIII binding to activated platelets

To determine if FXIII binds directly to GPIIb/IIIa receptor or receptor-bound fibrinogen is essential for FXIII binding to platelets, we studied platelet FXIII-A positivity when FXIII-A_2B_2 (25 μg/ml) with/or without γA/γ' fibrinogen (2.5 mg/ml) or γA/γA fibrinogen (2.5 mg/ml) were added to unstimulated and TRAP-activated (40 μM) washed human platelets for 15 minutes at 37°C in the presence of $CaCl_2$ (1 mM). Native human plasma fibrinogen containing well detectable amount of FXIII was used as a positive control to demonstrate FXIII-A positivity on TRAP-stimulated platelets. After incubation, samples were fixed in 1% PFA and then unbound FXIII-A_2B_2 with the excess of antibodies were removed by washing in PBS prior to analysis.

Statistical analysis

Unless otherwise indicated, data were expressed as mean ± SEM and analyzed by unpaired Student's *t* test. The p values less than 0.05 were considered significant.

In the DM study, Kolmogorov-Smirnov test was used for the evaluation of the normality of the data. Most outcome continuous parameters were non-normally distributed; therefore analyses were performed on log-transformed data for Student's independent *t* test analysis. Differences in various parameters among study groups were tested using analysis of variance and chi-square test as appropriate. Deviations from the Hardy-Weinberg equilibrium were analyzed using the chi-square test in each group. Multiple regression analysis was computed for checking the association of baseline characteristics with soluble P-selectin level. Univariate analysis of variance was used to adjust for significant variables and check for differences in soluble P-selectin levels within the different genotypes. Statistical analysis was performed using SPSS version 13.0.

RESULTS AND DISCUSSION

Significantly increased platelet and soluble P-selectin levels in obese and type 2 DM patients compared to healthy individuals

We investigated only the Thr715Pro P-selectin polymorphism; since it has been proved to be the most relevant to influence soluble P-selectin levels due to its localization. Regarding to all study individuals, there were marked differences in the baseline demographical and laboratory parameters and platelet activation markers between the study groups. Beside BMI, age also differed significantly between DM patients and healthy controls, since we could only enroll younger volunteers. Both the plasma level of soluble P-selectin and the percentage of platelet P-selectin were significantly increased in the BMI-matched non-DM obese subjects and in the type 2 DM group upon comparison with healthy individuals. Enhanced platelet reactivity was earlier demonstrated in both types of DM suggesting the causal relationship between severe angiopathy and activated platelets in children and adults.

Thr715Pro has minor effect on soluble P-selectin levels in the two patient groups

The frequency of Thr715Pro P-selectin genotype did not vary significantly between study groups. Healthy: 77.2% (AA, n=44), 22.8% (AC, n=13); BMI-matched non-DM: 81.3% (AA, n=39), 18.7% (AC, n=9); Type 2 DM: 74.8% (AA, n=89), 23.5% (AC, n=28), 1.7% (CC, n=2). All groups were in Hardy-Weinberg equilibrium. There was no subject with CC genotype in the healthy and BMI-matched non-DM obese groups. The CC genotype was rare in DM group, thus these subjects were pooled into the subgroup of patients with the AC genotype. In type 2 DM patients, the levels of soluble P-selectin were significantly increased compared to controls, but no difference ($p=0.642$) was observed between the two genotypes. In addition, although markedly lower soluble P-selectin levels could be measured in healthy Pro715 allele carriers, but the difference was not statistically significant ($p=0.060$). Moreover, in obese subjects, soluble P-selectin levels were also elevated compared to all healthy controls, but without difference ($p=0.777$) between AA and AC carriers.

Effect of BMI on soluble P-selectin levels in non-DM subjects

The association of BMI with soluble P-selectin levels was analyzed in all non-diabetic subjects. The levels of soluble P-selectin in healthy carriers of the C allele with BMI < 22.4 kg/m^2 were significantly (p=0.004) lower compared to controls with the AA genotype. Interestingly, this difference was not detectable in healthy subjects with higher BMI (≥ 22.4 kg/m^2). Although we could confirm previous reports that lower soluble P-selectin levels could be measured in healthy Pro715 allele carriers, but this was only true in lean subjects. Others also revealed a weak correlation between soluble P-selectin levels and BMI only in their healthy controls, but no association was found in CVD patients.

Effect of different variables on soluble P-selectin levels in type 2 DM patients

Previous studies have showed age- and sex-dependence of soluble P-selectin levels. However, another report found no correlation between the levels of soluble P-selectin and age in type 2 DM. In our DM group, age did affect the soluble P-selectin levels. There was a significant difference ($p<0.05$) in soluble P-selectin levels among older (median age of ≥54 years, 111.4±61.7 ng/ml) and younger (median age <54 years, 64.7±47.1 ng/ml) patients when all DM subjects were pooled, but genotype did not influence soluble P-selectin level even in these subgroups studied. Since age significantly affected soluble P-selectin levels, we compiled 2 age-matched groups and created 57 pairs; one consisting of DM patients and the other of healthy controls. P-selectin levels were significantly ($p<0.001$) higher in the DM group (mean: 79.4 ng/ml, quartiles: 39.7-121 ng/ml) as compared to the age matched healthy control group (mean: 46.6 ng/ml, quartiles: 33.2-57.6 ng/ml). The levels of soluble P-selectin did not vary with gender in the DM group either.

There was no statistically significant difference between male (91.5±65.5 ng/ml) and female (86.4±44.6 ng/ml) patients, and no variation could be demonstrated when the different sexes were analyzed according to genotype. The soluble P-selectin levels did not differ with the smoking status, since no statistically significant difference was found between 'never smoker' DM patients (87.8±49.7 ng/ml) and current DM smokers (90.9±80 ng/ml). Here, too, no difference was found in terms of soluble P-selectin levels according to genotypes. Some previous studies observed an association between Pro715 allele and lower soluble P-selectin levels, but this was true only in non-smokers with CVD. However, others failed to find this dependence in a healthy cohort.

In the DM group, multiple regression analysis was used to test for significant association between soluble P-selectin level and all the continuous variables. Only age was found to be significant in this response. After adjustment for this parameter along with the categorical variables (gender, blood pressure, smoking habit), no significant ($p=0.204$) difference was determined in the levels of soluble P-selectin by univariate analysis of variance among patients with AA and AC+CC genotypes. Future studies are required to investigate the significance of this polymorphism and the haplotypes of additional P-selectin polymorphisms in type 2 DM and in obesity.

Increased level of PMPs in patients after stenting

Stents represent a thrombogenic surface resulting in activation and deposition of platelets. Earlier, others investigated platelet P-selectin expression in PTCA patients receiving two different clopidogrel regimens with aspirin. They concluded that the measurement of platelet P-selectin was a sensitive tool to monitor the impact of angioplasty and the beneficial effect of antiplatelet drugs. Here, we applied blood sampling for PMP analysis at 15-minute time point after the intervention along with alternative platelet activation markers. Flow cytometry was assessed as the most widely used technique for the identification, quantitation and characterization of plasma MPs for its simplicity, and the wealth of information. By now, this tool has been standardized and optimized for MP detection. It is now generally accepted that only PS-exposing MPs i.e. Annexin V-positive events are considered as MPs, and PMPs are the most abundant form (80-95%), but the distribution of MPs with other cell origin is still a debate.

Thus, we determined the level of Annexin V-positive PMPs at the earliest time point that was technically feasible. Only one group published data at 15 minutes as the first sampling time point to measure PMP levels, where no significant increase of PMP levels was found by using ELISA. In contrast, we showed that there was significantly ($p<0.05$) higher level of PMPs ($557\pm83/\mu l$ versus $325\pm42/\mu l$) even by 15 minutes in patients with stent implantation compared to subjects with catheterization alone.

Elevated levels of other platelet activation markers in stented patients

As expected, the procedure of stenting induced activation of platelets demonstrating significantly higher levels of surface P-selectin ($2.7\pm0.3\%$ versus $1.99\pm0.1\%$) as it was found earlier. Interestingly, no soluble P-selectin level increase was

measured in stented patients compared to controls (38±12 ng/ml versus 36±11 ng/ml), which may be related to the early sampling time, and thus there was little time for surface P-selectin receptors to be shedded.

Although there was a tendency towards higher level of CD62 positive PMP population with higher CD62 MFI values, the difference in these results were not significant statistically between patients and control groups. Furthermore, the ratio of leukocyte-platelet heterotypic aggregates were also elevated, but only monocyte-platelet aggregates displayed a statistically significant increase after stenting (48±4% versus 38±3%). These findings are in agreement with some previous reports, which detected increased levels of PMPs in stented patients measured in coronary or peripheral circulation. However, we do not exclude the significance of MPs shedded from other source like endothelial cells. Although others found only mild endothelial injury after coronary angiography and PCI in stable angina patients when detecting the extent of vessel wall injury. The platelet-activating effect of stent implantation in patients could be compared more relevantly to those 'controls', whose coronary arteries were also exposed to the similar degree of invasive procedures during catheterization except for the stenting. This was evident from the data of these subjects who have elevated platelet P-selectin expression compared to former healthy controls in our DM study. Although stents may induce platelet activation, a follow-up study up to 30 days described a beneficial effect of stenting by detecting decreasing levels of PMPs and other platelet activation markers. Accordingly, the analysis of increased level of PMPs can be considered as an early and sensitive method to detect procedure-induced activation of platelets right after invasive cardiological interventions, and may supplement other tools like the measurement of surface CD63 expression, the plasma levels of soluble GPV, soluble CD40L and RANTES measured by ELISA to evaluate platelet reactivity. We used non-eluting bare metal stents; so further studies are needed to study if these parameters are suitable to predict prothrombotic events. In addition, the activating effect of other type of stents such as drug-eluting ones on platelet activation and MP levels for comparison to that of non-eluting type.

FXIII binds to TRAP-stimulated human platelets

We investigated the binding of non-activated plasma FXIII to human platelets in whole blood stimulated with TRAP. Platelet activation was tested by measuring P-selectin (CD62) labeling and the co-expression of these markers was studied subsequently on flow cytometer. In the absence of agonist, unstimulated platelets failed to bind FXIII. However, FXIII-A positivity significantly increased gradually upon platelet stimulation with TRAP in a concentration-dependent manner and reached the plateau level in response to 40 μM TRAP when mean CD62 positivity was over 90% indicating maximum platelet activation. No further increase in positivity of either marker was detected at higher TRAP concentrations. Furthermore, FXIII-A and P-selectin displayed a co-expression when platelets were activated with TRAP. These data suggest that FXIII is expressed only on stimulated human platelets. Similarly, a saturable and specific binding of FXIIIa to thrombin-stimulated, but not to resting platelets was demonstrated earlier.

Platelet-bound FXIII derives from plasma but not from platelet cytosol

It was not clear before if intracellular FXIII becomes available on the platelet surface upon activation. Former studies suggested that FXIII was retained during platelet activation. To determine whether platelet-bound FXIII derives from plasma or becomes released from intracellular localization and subsequently binds to the platelet surface, whole blood samples and washed human platelets were activated with increasing concentrations of TRAP and compared for FXIII-A and CD62 positivity. In washed platelet samples, no significant elevation in FXIII-A positivity was detected in samples stimulated with various concentrations of TRAP. However, human platelets in whole blood bound FXIII gradually in response to increasing agonist concentration. On the other hand, CD62 positivity significantly increased upon activation in both types of samples, which indicates that proper platelet activation occurred. The basal P-selectin level was much higher in unstimulated washed samples compared to control whole blood sample, since platelets were artificially activated during isolation in the washing procedure. According to these experiments, FXIII binds to platelets from plasma in whole blood, and the intracellular form is not expressed on platelets in TRAP-activated samples. Surprisingly, in a former report, gel-filtered human platelets when co-stimulated with collagen and thrombin (called COAT platelets) demonstrated enhanced surface expression of α-granule proteins as well as FXIII in the absence of exogenous FXIII.

Platelets with different size showed distinct levels of FXIII-A positivity

We further analyzed the FXIII-A and P-selectin positivity on large, medium and small platelets. Data were analyzed from experiments where whole blood platelets were activated by TRAP. We found that large platelets displayed the highest FXIII-A positivity, while small platelets showed the lowest level. However, activated platelets were equally positive for P-selectin expression regardless of platelet size. These data suggest that the binding site for FXIII may be a platelet receptor that correlates with platelet volume.

Eptifibatide and RGDS-tetrapeptide significantly inhibited FXIII-A positivity on stimulated platelets

Activated platelets express approximately 80 000 copies of GPIIb/IIIa receptors on their surface and this activation-dependent receptor plays a significant role in fibrin(ogen) binding during platelet activation. Moreover, inhibition of these receptors by using $F(ab')_2$ fragments of the monoclonal antibody (7E3) reduced markedly the arterial eversion graft thrombosis and *ex vivo* platelet aggregation in dogs. Here, we investigated the role of GPIIb/IIIa receptor in FXIII binding to TRAP-stimulated platelets in whole blood using pharmacological agents against this receptor. Eptifibatide was used as a highly specific GPIIb/IIIa receptor antagonist at the typical concentration (2 μg/ml) as being administered during emergency cardiological intervention. Platelets were stimulated with different concentrations of TRAP after 15 min of preincubation with eptifibatide. Fifty-five % of FXIII binding was prevented by eptifibatide treatment compared to the untreated activated sample ($p<0.05$), but FXIII binding was not totally abolished. When platelets were pre-treated with RGDS tetrapeptide, the inhibition of fibrinogen binding to platelets resulted in even lower levels of FXIII binding. FXIII-A positivity was inhibited by 73% in the presence of RGDS peptide versus control sample. Platelets were properly activated by TRAP as measured by the P-selectin expression. These results suggest that GPIIb/IIIa receptor and receptor-bound fibrinogen have an important role in FXIII binding. Earlier data on the binding site of FXIIIa were contradictory. In the first study, GPIIb/IIIa receptor and platelet-bound fibrin(ogen) were excluded as binding site for FXIIIa as RGD peptide did not block the interaction between FXIIIa and platelets. The binding of FXIIIa to thrombin-activated platelets was inhibited by plasmin. In a later study, the GPIIb/IIIa receptor was shown to mediate the binding of FXIIIa to thrombin-stimulated platelets.

Impaired binding of non-active FXIII to activated GT platelets

In order to support the theory that GPIIb/IIIa receptor is involved in FXIII binding, we analyzed the FXIII-A positivity on TRAP-activated platelets in whole blood from a patient with GT. Simultaneously, platelets were also examined from age-matched healthy controls. No difference in basal FXIII-A positivity was found between the unstimulated normal and GT samples. However, upon TRAP-induced platelet activation, insignificant FXIII-A positivity was measured on GT platelets compared to healthy platelets. Normal and GT platelets were both properly activated by TRAP as evidenced by the elevated P-selectin values. These data confirm our previous findings that GPIIb/IIIa receptor has a pivotal role in FXIII binding during platelet activation. Surprisingly, FXIIIa binding to stimulated platelets from two patients with severe Glanzmann thrombasthenia was found to be normal.

FXIII-A_2B_2 requires fibrinogen with γ'-chain to bind to activated platelets

The next question was whether FXIII binds directly to GPIIb/IIIa receptor or the presence of fibrinogen is essential for FXIII binding. To study the characteristics of FXIII binding process, we used two different variants of fibrinogen with distinct molecular structure. Fibrinogen molecules are comprised of two sets of three polypeptide chains termed Aα, Bβ and γ chain. A splicing variant of the γ-chain, termed the γ'-chain, which is present only in approximately 15% of fibrinogen molecules was found to bind strongly FXIII via its B subunits. Sample with native plasma fibrinogen containing FXIII was used as a positive control showing significantly higher FXIII-A positivity compared to control sample ($p<0.01$). TRAP-activated platelets displayed only slightly increased FXIII-A positivity when exogenous plasma FXIII-A_2B_2, or either variant of fibrinogen (γA/γA or γA/γ') with minor FXIII content was added alone in samples. However, γA/γ' fibrinogen in the presence of non-active FXIII-A_2B_2 significantly ($p<0.05$) augmented FXIII binding on TRAP-activated platelets compared to control sample and platelets treated with γA/γ' fibrinogen only. On the other hand, washed platelets incubated with FXIII-A_2B_2 in the presence of γA/γA fibrinogen showed only insignificant ($p=0.302$) increase in FXIII-A positivity as compared to platelet sample containing γA/γA fibrinogen alone. These data suggest that FXIII-A_2B_2 from plasma binds to thrombin-receptor activated human platelets

via GPIIb/IIIa receptor-bound fibrinogen containing γ'-chain and it is not capable of direct platelet binding to the fibrinogen receptor.

This is in agreement with a previous report where it was shown that γA/γ' fibrinogen binds FXIII 20-fold more tightly than γA/γA fibrinogen. Other group also demonstrated the indirect binding of FXIIIa through fibrinogen associated with its receptor. However, they did not provide more data about the mechanism of FXIII binding. This mechanism targets FXIII to the surface of stimulated platelets where thrombin and consequently fibrin is formed and FXIII becomes activated, and cross-linking of fibrin by FXIIIa becomes promoted on the surface of activated platelets.

It was to be stressed that throughout our study non-activated plasma FXIII was used. FXIIIa only consists of thrombin cleaved A subunits without the dissociated B subunits, while, as demonstrated above, plasma FXIII seems to bind to stimulated platelets through the B subunits. Therefore, the binding sites for FXIII and FXIIIa must be distinct and our results are not comparable with the earlier findings.

It is interesting that in a clinical study in patients with PAD platelet-associated FXIII was found significantly higher than in healthy controls, and the detection of FXIII on platelets was proposed as a marker of platelet activation. We also suggest that the detection of FXIII binding to active platelets may be a useful alternative platelet activation marker during *ex vivo* sample analysis.

SUMMARY

Cardiovascular and cerebrovascular diseases are still the leading cause of death in the developed world. Activated platelets are fundamentally involved in the pathomechanism of thrombotic complications in these diseases. That is why the investigation of activated platelets has become more obvious in the daily routine. Our primary goal was to detect increased platelet activation in time that occurs often in acute or chronic vascular disorders or induced by invasive therapeutic intervention in such states.

In this present study, we found that in type 2 DM and obese patients, platelet and soluble P-selectin levels were significantly elevated compared to the findings in healthy controls, but the most studied P-selectin gene polymorphism (Thr715Pro) did not affect plasma soluble P-selectin values in the patient groups. In DM patients divided into different subgroups according to several demographical variables, the levels of soluble P-selectin still did not vary notably according to their genotype for this polymorphism.

In patients with stable angina, significantly increased levels of PMPs, platelet P-selectin and platelet-monocyte aggregates were measured compared to angina patients underwent catheterization alone. However, soluble P-selectin levels did not show marked difference between the two study groups. Thus, the measurement of PMP levels can be considered as an early sensitive activation marker to detect platelet activity right after invasive cardiological interventions.

In vitro, FXIII-A_2 was not expressed from its intracellular localization on washed platelets activated by TRAP. Thus, surface-bound FXIII on stimulated whole blood platelets is of plasma origin. The presence of γA/γ' fibrinogen significantly potentiated the binding of purified FXIII-A_2B_2 on stimulated washed platelets, but no FXIII-A positivity was seen without this type of fibrinogen or with fibrinogen having γA-chain only. Accordingly, plasma FXIII is unable to bind directly to the activated platelet surface, and significant binding of non-active FXIII occurs only when GPIIb/IIIa receptor-bound fibrinogen with γ'-chain is present. Analysis of FXIII binding to platelets may be an additional sensitive activation marker in the future.

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