EVALUATION OF A GLOBAL PLATELET FUNCTION TEST (PFA-100 CLOSURE TIME) IN DIAGNOSTICS OF PLATELET FUNCTION DISORDERS

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INTRODUCTION

Platelets play a basic role in haemostasis, their functions are supported by several complex biochemical mechanisms. Adhesion of platelets to damaged wall of blood vessels, their aggregation, followed by the secretion of molecules critically important for haemostasis from platelet granules and the procoagulant effect of the activated platelet surfaces are all parts of a complex pathway. The platelet functional disorders could be categorized according to the steps leading to platelet activation.

The von Willebrand’s disease (vWD) is the most common congenital platelet function disorder, characterized by the quantitative or qualitative disorder of the von Willebrand’s faktor (vWF). The vWF binds to collagen (and other subendothelial structures) uncovered by the injury of the vessel wall, and to its receptor, the glycoprotein Ib-IX complex (GPIb-IX) present on the surface of platelets, thus ensuring the adhesion of platelets to the injured vessel wall. Under high shear flow conditions vWF also binds to another platelet receptor GPIIb-IIIa („fibrinogen receptor”), thus a vWF multimer is essential for platelet aggregation as well. Another basic biological function of vWF is that it serves as a carrier protein for coagulation factor VIII (FVIII), protecting it from proteolytic degradation.

There are three major types of vWD. Type 1 is characterized by lower level of vWF, but the multimer structure of vWF is normal. Type 2 refers to a group of distinct abnormalities of vWF function due to modified multimer structures. In this group several subtypes could be distinguished. In subtype 2A the functional disorder of vWF is associated with the absence of large molecular mass multimers; in subtype 2M beside the abnormal function (reduced binding to platelets) the multimer structure is intact. In the type 2B vWD the affinity of vWF to GPIb receptor is increased, and as a consequence the large molecular mass multimers disappear from the plasma due to their increased binding to platelets. Elevated response of platelets to ristocetin is characteristic for this type.
In subtype 2N binding of vWF to Factor VIII is abnormal, and as a consequence normal von Willebrand’s factor antigen level and ristocetin cofactor activity are associated with low FVIII level. The type 3, recessively inherited vWD is the most serious form, since this disorder is characterized by practically the complete absence of vWF.

The Bernard-Soulier syndrome, caused by the absence of the vWF receptor GPIb-IX on platelets, or its defect leading to a reduced affinity toward vWF binding also results in adhesion defects of platelets. A rare and unusual defect in GPIb-IX could lead to the so called platelet type pseudo-vWD. Like in subtype 2B vWD the binding of vWF to its receptor is elevated and as a consequence the large molecular mass multimers are missing from the plasma, however the enhanced binding is due to the abnormality of GPIb-IX and not to that of vWF. A defect in the direct platelet collagen receptor (GPIa-IIa) could also lead to adhesion defects, since it is also required for the attachment to the injured vessel wall.

The next step in platelet activation is the aggregation of platelets, during this step the activated platelets will form aggregates through the fibrinogen present in the plasma. The potential fibrinogen receptor GPIIb-IIIa complex does not bind to fibrinogen on dormant platelets. In order to induce the binding ability of the fibrinogen receptor, the biochemical mechanisms triggered by the binding of platelet activating agents [the so called primer agonists: ADP, thrombin, thromboxan A$_2$ (TXA$_2$)] to their receptors is required. Defects in the receptors of these primary agonists also result in aggregation abnormalities, but these defects – except the defect in the ADP receptor – cause secretion abnormalities too. The causative agents of aggregation abnormalities are usually afibrinogenaemia (lack of fibrinogen), dysfibrinogenaemia (abnormal fibrinogen structure) or the Glanzmann thrombasthenia. This last disease is the result of a defect in the fibrinogen receptor GPIIb-IIIa.
Behind the platelet secretion abnormalities are biochemical mechanisms, storage pool diseases or defects of adenine nucleotide metabolism. The inherited abnormalities of biochemical mechanisms are caused by enzyme defects (phospholipase, cyclooxygenase, thromboxan synthetase), or defects in the above mentioned receptors or signal transduction pathways. Most frequently cyclooxygenase or thromboxan synthetase inhibitors are the causative agents of the acquired abnormalities.

Storage pool diseases in megakaryocytes appear to result from the failure of granule formation or the formation of functionally defective granules. Storage pool diseases are categorized into three groups. The δ storage pool disease could be present alone, or could be associated with other inherited diseases. Examples for this last group are the Hermansky-Pudlak, Chédiak-Higashi, Wiskott-Aldrich and TAR syndromes. In αδ storage pool diseases the α and δ granules of platelets are missing or abnormal. The gray platelet syndrome belongs to the third group, which involves the α storage pool diseases.

Scott syndrome is a disorder of the procoagulant activity of platelets. The diagnosis of abnormalities leading to defective platelet adhesion, aggregation, secretion and procoagulant activity requires a number of complicated, expensive laboratory tests that usually are carried out in diagnostic centers. There has been a need for a screening test that covers the complexity of platelet function, and detects the majority of diseases with impaired platelet function. Bleeding time has been considered as the most appropriate screening test for detecting disorders of platelet function. This screening test – with the rare exceptions of disorders of the procoagulant effect – provides a complex picture about platelet functions, but it is imprecise and insensitive. A further problem is that it is very difficult to standardize this test, and in case of infants or children it is executable only with difficulties. Overall, the bleeding time test is impractical for the patient, it cannot be used in serial investigations and its predictive value for surgical bleeding has also been criticized.
Since anti-platelet agents are widely used to prevent atherothrombotic events, it would be very important to monitor their effect on platelet function in serial investigations. Based on these notions, we can conclude, that there is a serious need for an ex vivo test to study the complexity of platelet functions, that is suitable for monitoring and for serial investigations too. Such a test has to imitate the in vivo conditions as closely as possible and has to be sensitive enough to detect minor platelet function disorders.

Recently, a new platelet function analyzer, PFA-100 (Dade-Behring, Deerfield, IL, USA), was recommended for assessing impaired platelet function ex vivo. By PFA-100 the complexity of platelet function is tested at a high shear rate (5,000-6,000 sec\(^{-1}\)) that corresponds to flow conditions present in small arteries. The citrated blood sample (0.8 ml) is aspirated under vacuum from the reservoir of a cartridge and it flows through the aperture of a membrane. The membrane is coated with combinations of platelet agonists, either with collagen/epinephrine (CEPI) or with collagen/ADP (CADP). Platelets adhere to the membrane surrounding the aperture and progressively a platelet aggregate is formed that finally occludes the aperture. The time required for the complete occlusion is recorded as closure time (CT) by the instrument. PFA-100 closure times are not influenced by therapeutic concentration of heparin or nitroglycerin.

Another general ex vivo platelet function test is the O’Brian filter method. In case of von Willebrand’s patients our results obtained with the PFA-100 platelet function analyzer were compared to results obtained with the O’Brien filter method by Dr. Ágota Schlammadinger and her coworkers.

AIM OF THE STUDY

The aim of the present study was to provide further data on the value of PFA-100 for screening patients with vWD and with inherited and acquired platelet
defects (at the time of our investigation there were only sporadic publications about its usefulness in diagnostic investigations) and compare the performance of PFA-100 with the template bleeding time test. Four major fields of this topic were investigated:

1/ Diagnostics of vWD with defects in platelet adhesion and aggregation, a field where there were no suitable screening tests available.

2/ Diagnostics of some of the rare inherited platelet aggregation and secretion disorders (Glanzmann thrombasthenia, Hermansky-Pudlak syndrome)

3/ The usefulness of PFA-100 in diagnostics of the acquired secretion disorder caused by the therapeutically administered acetylsalicylic acid (ASA); and in monitoring the effectiveness of ASA.

4/ Detection of platelet functional disorders provoked by thrombolytic therapy and the prediction of possible dangers of bleeding complications, based on the detected platelet disorders.

PATIENTS, MATERIAL AND METHODS

Determination of plasma coagulation tests, coagulant factors and fibrinogen level

Plasma was separated by centrifugation (2000 x g, 20 min, at 22 °C) of citrated blood. Plasma coagulation tests (except reptilase time), coagulant factors and fibrinogen level were determined by STA Compact (Diagnostica Stago, Asnieres, France) coagulometer. Prothrombin time (PT) was measured by Dade Innovin (Baxter Diagnostics Inc., Deerfield, USA) and activated partial thromboplastine time (APTT) was measured by PTT-automate (Diagnostica
Stago, Asnieres, France). Reptilase time was determined by KC 1A (Amelung, Lemgo, Germany) coagulometer with F.T.H. 50-Reptilase reagent (Diagnostica Stago, Asnieres, France). The activity of coagulant factors (FVIII, FIX, FXI, FXII) were performed by one stage clotting assay based on the APTT using deficient plasma from Stago Diagnostica (Asnieres, France). Determination of thrombin time (TT) and the plasma fibrinogen determination by the Clauss method were performed by using Reanal (Budapest) reagents.

Determination of template bleeding time
Template bleeding time was determined by Simplate II R disposable device. A reference interval of 2.5-9.5 min was calculated for the template bleeding time by the manufacturer.

Determination of the reference interval of PFA-100 Closure Time
For PFA determination blood was drawn into vacutainer tubes containing buffered citrate. The control group consisted of 31 healthy individuals. Closure time (CT) determinations by both cartridges were carried out within 30 minutes. The reference interval for both (collagen/epinephrine (CEPI) and collagen/ADP (CADP) cartridges was determined from the measured values of the control group.

Determination of platelet aggregation and secretion
Platelet agonist (ADP (10 μmol/L), collagen (1 and 5 mg/L), epinephrine (10 mg/L), arachidonic acid (500 μg/mL) induced platelet aggregation and secretion and thrombin (5 U/mL) induced ATP secretion were performed in Chrono-log lumiagggregometer. The amount of ATP released from dens granules were measured by a bioluminescent method.
Flow cytometric analysis
In order to prove the diagnosis of Glanzmann thrombasthenia, platelets were analyzed by forward and side light scatter and by the detection of the expression of GPIb/IX receptor on the surface of platelets by a Becton Dickinson FacScan Analyzer.
A fluorescein-isothiocyanide (FITC) labeled monoclonal antibody (CD42a) was used for determination of GPIb/IX receptor complex. Phycoerythrin and FITC labeled monoclonal antibodies were used for the flow cytometric determination of platelet membrane glycoproteins IIb (CD41) and IIIa (CD61), respectively.

Laboratory diagnosis of vWD
The diagnosis and classification of vWD was based on the further tests:
Platelet agglutination induced by different concentrations (0,6 mg/mL és 1,2 mg/mL) of ristocetin (RIPA) were carried out in Chrono-Log lumiaggregometer. Determination of vWFAg (vWF:Ag) was conducted by immunturbidimetric method. The vWF ristocetin cofactor activity (vWF R:Co) was determined by a commercially available kit from Helena according to the description of the manufacturer. The molecular weight based analysis of the multimeric structure of vWF was carried out by sodium dodecyl-sulphate (SDS) agarose gel electrophoresis. FVIII activity was determined by APTT based one stage assay.
32 patients with vWD were included in the study. Among the patients with vWD the following distribution of subtypes was found (based on family and personal bleeding history and on the results of laboratory tests): type 1, n=22; type 2A, n=2; type 2B, n=4; type 2N, n=1; type 3 n=3.

Laboratory diagnosis of Hermansky Pudlak syndrome
10 albino children (age 7-14) were screened for the occurrence of HPS.
The detailed analysis of platelet functions of albino children were published by Pap and co-workers, members of our institute. Beside the lumiaggregometric
analysis of platelets the diagnosis was based on the following tests: ADP and ATP content of platelets was measured by a bioluminescent method, the number of dense bodies in platelets was determined by mepacrine uptake test and by flow cytometric determination of CD63, a dense body membrane protein.

10 albino patients, 5 with and 5 without HPS, and one patient with Glanzmann thrombasthenia were also included in the study. Additionally to bleeding time and PFA-100 closure time in blood samples of these patients we have also determined the platelet counts, and the necessary diagnostic tests to prove the diagnosis of the disease (see above).

Evaluation of the effect of ASA on PFA-100 closure time
The study was carried out on blood samples of 4 volunteers working in our laboratory. They got a single dose of 500 mg Aspirin (Bayer). Determination of the bleeding times, PFA-100 closure times and platelet aggregation and secretion investigations were carried out before and 24 hours after the administration of ASA. Platelet count was also determined in each sample. In case of two individuals the same tests – except bleeding time – were repeated at later times.

Evaluation of the effect of streptokinase on PFA-100 closure time
The in vitro effect of streptokinase (SK) on PFA-100 closure times was investigated on blood samples from seven non-medicated healthy individuals. The closure times of all untreated blood samples were in the reference intervals. The samples were treated with 50 U/mL or 250 U/mL SK for 4 hours at 37 °C. Control samples were incubated with identical volume of physiological saline. Following incubation aliquots of anticoagulated blood were removed for platelet count determination and for measuring PFA-100 closure time in CEPI and CADP cartridges. From the remaining blood plasma was separated by
centrifugation (2,000 x g, 20 min, at 22 °C) and thrombin time was also determined.

33 MI patients undergoing thrombolytic therapy were involved in the study. Following diagnosis patients received a total dose of 1.5 million Units SK intravenously and 250 mg ASA orally. The thrombolytic therapy started with an intravenous bolus of 250,000 Units SK that was followed by continuous SK infusion for one hour. Unfractionated heparin infusion (1000 U/hr) was started two hours after the initiation of SK therapy. Blood sample was drawn from the patients before, 2 hours and 4-6 hours after the start of thrombolytic therapy and basic coagulation tests, and platelet count were determined. The onset of “lytic state” was demonstrated in all patients by the significant prolongation of thrombin time, activated partial thromboplastin time and prothrombin time two hours after the start of SK therapy. In none of the patients was a significant change in the platelet count during the investigation period.

RESULTS

Determination of the reference range of PFA-100 closure time

In our study we have determined the closure time using both cartridges in 31 healthy individuals showing normal results in homeostasis screening tests. The measured values showed normal distribution and as a result (x±2 SD) 63-142-second and 55-118-second reference intervals were obtained for closure time with collagen/epinephrine and collagen/ADP cartridge respectively.

Evaluation of the value of PFA-100 platelet function analyzer for screening in the diagnostic of vWD patients

Comparison of Bleeding Times and PFA-100 Closure Times measured with the collagen/epinephrine cartridge

In the case of vWD patients with prolonged bleeding time (BT: ≥ 10 min) the CT measured with the collagen/epinephrine cartridge was uniformly prolonged.
9 patients with type 1 disease, the type 2A patients, one of the type 2B patients and all three type 3 patients belonged to this group. The high number of patients with no closure at all is to be noted. The second group consisted of patients with BT in the upper reference interval (BT: 7.5-9.5 min). 80% of these patients also demonstrated prolonged CT or no closure at all. One of the two patients with no CT prolongation was the patient considered a 2N variant. Seven patients (five type 1 and two type 2B) with BT in the lower reference range (BT: ≤ 7.0 min) belonged to the third group. Three type 1 patients had prolonged CT with the collagen/epinephrine cartridge, while the rest, including the two mild IIB variants, had CT within the reference interval. The overall sensitivity of the BT test in the case of vWD patients was rather poor (48.4%) when the 2N variant was omitted. The sensitivity of CT with collagen/epinephrine cartridge was much higher (83.9%). This test missed only a 2N variant and a limited number of mild cases of type 1 and type 2B disease.

Comparison of Bleeding Times and PFA-100 Closure Times measured with the collagen/ADP cartridge

The results with the collagen/ADP cartridge were also divided into three groups. With the exception of three type 1 patients, the patients with prolonged BT had no closure with this cartridge. The two patients with normal CT had their BT only slightly above the reference range (10.0 and 11.5 min). In the second group (BT: 7.5-9.5 min) two of the three patients with normal collagen/ADP CT also had normal CT with the collagen/epinephrine cartridge. In the third group (BT: ≤ 7 min) only 2 out of 7 patients had prolonged collagen/ADP CT. The overall sensitivity with the collagen/ADP cartridge was 74.2 %.

Previously in a collaboration with our group, Ágota Schlammadinger and Zoltán Boda (II. Clinic of Internal Medicine; University of Debrecen) compared the PFA-100 closure times with the results obtained with the O’Brian filter tests in the case of vWD patients. The two tests gave similar results, sensitivity of the
O’Brian filter test with citrated blood samples was proved to be between 82.1 and 89.3% considering the different retention and blocking drop count parameters.

Evaluation of the value of PFA-100 closure times in the diagnostics of other inherited platelet function disorders (Glanzmann thrombasthenia, Hermansky-Pudlak syndrome)

*Laboratory diagnostics of a patient with Glanzmann thrombasthenia*

A six month old child was investigated for bleeding diathesis. He demonstrated umbilical bleeding and hematoma at the site of intramuscular injection after birth. At the age of six months he was admitted to hospital due to operating his inguinal hernia. After admittance to the clinic a hematoma at the site of intramuscular injection, and severe epistaxis were developed in the patient. Since then – the time of diagnosis – he has had repeated episodes of severe bleeding. There was no family history of bleeding diathesis.

Platelet count was in the reference range, while a highly significant prolongation of bleeding time was verified (> 20 min). Highly prolonged PFA-100 closure times were measured both with collagen/epinephrine and collagen/ADP cartridges. Among the screening tests for coagulopathies the APTT showed significant prolongation but no other screening tests showed substantial abnormality. In most cases the coexisting prolongation of bleeding time and APTT suggests a platelet adhesion disorder. We have to add that the von Willebrand’s factor is also a carrier molecule for Factor VIII. In the patient it was possible to correct the APTT with normal plasma. Activity of the prephase coagulation factors has also been measured. The results showed, that APTT was prolonged due to the slight decrease in the level of coagulation factors IX and XI. These values, however, do not explain the severe bleeding disorder of the patient. Besides normal Factor VIII level the level of von Willebrand’s factor antigen was 130% (the reference range is 50-160%), the
ristocetin cofactor activity was 78% (reference range 50-160%), these values exclude the possibility of von Willebrand’s disease.

High dose ristocetin was able to induce aggregation, however, even in this case an unusual irreversible form of aggregation response was observed. Investigations using other aggregating agents showed some morphological changes of the patient’s platelets – showing that the agent binds to its receptor – but no aggregation response was observed when the patient’s platelets were activated with the usual activating agents. ADP failed to induce release reaction, while collagen and arachidonic acid induced only a slight release of ATP, which is a secondary consequence of the lack of aggregation. Nearly normal ATP secretion was induced by high dose thrombin which is a strong inducer of aggregation independent secretion. Results of the platelet aggregation and secretion studies unambiguously showed a platelet aggregation disorder. During aggregation platelets are attached together by fibrinogen, so in case of afibrinogenanemia and some cases of dysfibrinogenemia there is no aggregation.

The patient’s fibrinogen concentration was normal and the reptilase time – being sensitive to dysfibrinogenemia – showed only minor prolongation. These results together suggested a defect of the fibrinogen receptor, typical to Glanzmann thrombasthenia.

The diagnosis was further supported by flow cytometric investigations which verified a highly significant decrease of GPII on the surface of patient’s platelets. A fluorescein-labeled anti-GPIX antibody – GPIX is a subunit of the receptor of von Willebrand’s factor – reacted strongly with platelets of the control sample and that of the patient. However, the phycoerythrin-labeled anti-GPIIb antibody reacted only with the control platelets. Similar results were obtained with the fluorescein-labeled anti-GPIIb-IIIa (fibrinogen receptor) antibody. These results clearly indicate that the patient is suffering in Glanzmann thrombasthenia.

Further molecular genetic and protein biochemical analysis of the case was carried out by our coworker Gergely Losonczy, who showed, that the mutation
leading to the disease (a single nucleotide deletion resulting in a frameshift and an early stop codon as a consequence) is in the gene coding for the GPIIb protein. The detailed description of his work can be found in his PhD thesis.

PFA-100 Closure Times in Hermansky-Pudlak Syndrome

Previously Pap and his coworkers from our institute published the investigation of platelet function disorders in albino children. The 5 albino children with established HPS (albinism, platelet storage pool disease, ceroid-like pigment deposition in the bone marrow or in RES cells) and with mild to moderate bleeding tendency had uniformly highly prolonged bleeding time. Closure time measured with the collagen/epinephrine cartridge showed prolongation in 3 cases and no closure was observed in an additional child. One child had CT in the upper normal range. In none of the children was prolonged CT observed with the collagen/ADP cartridge.

Evaluation of the effect of acetylsalicylic acid (ASA) treatment on PFA-100 closure time

During the investigations of the 4 individuals who got a single 500 mg dose of Aspirin we can conclude that the bleeding time just barely reacts to Aspirin, similarly with collagen/ADP cartridges we have not found drastic changes in closure times. In the collagen/epinephrine stimulated system, however ASA administration caused significantly prolonged closure time. In two of these cases prolonged closure time meant no closure even after 300 sec. In one individual despite of the administration of a large single dose of Aspirin the closure time was prolonged to 142 seconds only, which suggests that there may be significant individual variability in the response to ASA.

Using the traditional aggregometric or lumiaggregometric methods to determine the effect of Aspirin we have found, that ADP aggregation did not decrease
drastically, however the epinephrine induced aggregation became single phased and collagen and arachidonic acid induced aggregation reduced by Aspirin. In the case of Aspirin treated individuals ATP secretion showed a decrease or it was absent.

Results obtained with collagen and epinephrine stimulated (CEPI CT) samples showed good correlation with the uniform, drastic decrease in ATP secretion. Aggregation is less sensitive to ASA than secretion. ADP aggregation showed only a minor decrease, however the low dose collagen, arachidonic acid and epinephrine induced aggregation proved to be a better indicator of the effect of ASA.

In order to follow the time dependence of ASA effect PFA-closure times were determined at further, additional times in the case of two Aspirin treated individuals. Only the collagen/epinephrine cartridge showed the effect of ASA, but it was very sensitive even after four hours. The effect of ASA was only hardly detectable after 3-4 days of administration.

Evaluation of the effect of streptokinase on PFA-100 closure time

Impaired platelet function plays a major role in the bleeding episodes during thrombolysis, behind this phenomenon is the damage in platelet functions which is a consequence of the thrombolysis. The purpose of our study was to evaluate if PFA-100 could be used for monitoring platelet function during thrombolysis. Before the clinical studies we have carried out in vitro experiments.

In vitro addition of 50 U/mL SK to whole blood caused a >30 sec prolongation of CADP closure time in one out of seven blood samples taken from healthy individuals. A 5-times higher dose of SK also prolonged the CADP closure time in two additional blood samples. SK at concentrations of 50 and 250 U/mL induced the prolongation of CEPI closure time by more than 100 sec in four and five blood samples, respectively. In none of the blood samples was shortening of closures times observed. The effective activation of fibrinolysis was
demonstrated by the considerable prolongation of thrombin time in all cases. Platelet count did not change significantly during the experiment. The results of in vitro experiments demonstrated that platelet function defect caused by the activation of fibrinolysis could be detected by measuring PFA-100 closure time and suggested that this technique can be used for the detection of impaired platelet function during thrombolytic therapy.

Among the 33 MI patients investigated by PFA-100 only a single patient demonstrated prolonged closure time (132 sec) with the CADP cartridge. During the course of thrombolytic therapy the CADP closure time remained unchanged or its prolongation was insignificant (0-30 sec) in two third of the patients. In eleven patients a more than 30 sec (range: 31-97 sec) prolongation of CADP closure time was induced by the thrombolytic therapy. In 2 of these cases, however the closure time still remained in the reference interval. It is to be noted that CADP closure time, in contrast to CEPI closure time, is unaffected by ASA and its prolongation represents an impaired platelet function caused by factors other than ASA.

It has been shown that CEPI closure time is highly sensitive to the effect of ASA and its prolongation is a reliable indicator of the therapeutic response and the patient’s compliance. Seven out of the 33 patients were on long term ASA treatment prior to the onset of MI. All seven patients had CEPI closure times above the reference interval before the administration of ASA and SK, and 5 of them had CEPI closure time >300 sec. It is interesting that in three patients the originally >300 sec CEPI closure time temporarily decreased, and then, after 4 hours, returned to the pre-thrombolysis value. 15 out of 26 patients not being on ASA therapy before the onset of MI (58%) responded to ASA with a higher than 120 sec prolongation of CEPI closure time (responders) and in most of these cases the closure time exceeded 250 sec. About half of these responders (7 out of 15 patients) showed an early response that reached maximum within 2 hours, while in other cases it took 4 hours to obtain maximal response. The
closure time of 5 patients out of 26 remained in the reference interval and there were two additional patients with somewhat prolonged pretreatment CEPI closure times (187 and 170 sec) who also failed to respond to ASA. Thus, the total number of non-responders (prolongation 0-50 sec) was 7 (27%). In 4 patients the prolongation was between 51-120 sec and the CEPI closure time remained below 250 sec. Patients in this group (15%) could be considered as semi-responders.

DISCUSSION

PFA-100 closure time in von Willebrand’s disease patients

In diagnostics of congenital bleeding disorders most of the problems are caused by the von Willebrand’s disease (vWD) and its subtypes. The blood type dependence of the vWF level and its temporal changes make it difficult to evaluate the diagnosis of this disease showing usually mild or hardly detectable symptoms. The vWD, however could cause serious bleeding complications during giving birth or going through surgery. In a study involving large number of healthy individuals it was found, that in individuals belonging to blood group 0 the level of vWF is lower than that of in individuals with blood type A, B or AB. It is interesting to note, that more than 65% of vWD patients belong to blood group 0.

Diagnostics and classification of vWD are time consuming and expensive processes involving a complex series of laboratory tests. The necessary tests for diagnosis have to involve at least the determination of vWF antigen, vWF R:Co activity and FVIII activity, in some cases just for the right diagnosis these tests have to be supplemented with tests necessary for classification, such as the ristocetin aggregation, vWF multimer determination, determination of collagen binding capacity, mixed aggregation tests, vWF receptor studies; just to mention a few important tests. The results of laboratory tests especially in the case of
type 1 and type 2 vWD subgroups very often do not correlate with the severity of clinical symptoms. There is a need for a screening test that properly reflects the severity of bleeding disorders.

In our study the sensitivity of BT in detecting vWD was 48.4% which is lower than in the two other studies, 65.5% and 59.1%. The slight discrepancy might reflect the difference in the severity of vWD among the patients involved in the three studies.

A recent study investigated the performance of PFA-100 CT as a screening test for vWD on 60 patients with different subtypes of the disease. The overall sensitivity of CT test with the collagen/epinephrine cartridge was comparable with our results (96.5% versus 83.9%) and only a few mild cases were missed by the test. In another report 95.5% diagnostic sensitivity was established in a group of patients 44 with vWD. By the collagen/ADP cartridge, however, somewhat discordant results were obtained. The 100% sensitivity observed by Fressinaud et al. could not be reproduced with our patients. Instead, the sensitivity obtained with the collagen/ADP cartridge, was lower (74.2 %) than that obtained with the collagen/epinephrine cartridge. 3 patients with vWFAg between 0.28-0.31 U/mL, for instance, had collagen/ADP CT in the reference range, although in its upper quartile.

Our results on type 1 of vWD are comparable with those of Fressinaud et al. In types 2A, and type 3 no closure was observed with either of the cartridges in both studies, while in type 2N CTs were in the reference range. The results in type 2B vWD are less uniform. In our study two patients out of four, in the other study two patients out of three had infinite closure times. These patients had vWF R:Co activity below the reference range and one patient in both studies had decreased platelet count. In our study two type 2B patients had normal vWF R:Co activity and normal platelet count with BT and CTs in the reference interval. Fressinaud et al. had one type 2B patient with vWF R:Co in the reference interval whose CT in the collagen/epinephrine cartridge was only
slightly prolonged. The results suggest that in type 2B vWD PFA-100 closure times may widely vary depending on the actual level of functional vWF.

To summaries our results we can conclude that CT measured by both cartridges was superior to BT for screening vWD. Based on our results it would be advisable that the PFA-100 closure time determination could be included in the diagnostic panel when vWD is suspected. The superiority of PFA-100 CT over template BT was especially evident in the patients with mild to moderate type 1 vWD. The PFA-100 closure time is also useful for monitoring DDAVP therapy in patients with type 1 von Willebrand’s disease, because the DDVAP therapy corrects the initially prolonged CT of patients.

Evaluation of the value of PFA-100 closure times in the diagnostics of other inherited platelet function disorders (Glanzmann thrombasthenia, Hermansky-Pudlak syndrome)

Glanzmann thrombasthenia

Platelet count was in the reference interval, while a highly significant bleeding time was verified. These results suggested severe platelet function disorders. This diagnosis was further supported by the highly prolonged PFA-100 closure times measured with both collagen/epinephrine and collagen/ ADP cartridges. On the aggregation curves the signal attributed to changes in the shape of platelets is clearly visible (a temporary drop in light transmission), meaning that the aggregation agents were bonded to the platelet membrane and activated the biochemical mechanisms needed for the morphological change. The aggregating agents, however were not able to activate aggregation.

In case of those agents (arachidonic acid, thrombin, large dose collagen), where aggregation has no role in supporting secretion, the biochemical mechanisms triggering secretion came into action and nearly normal level of ATP secretion was measured.
Considering that the patient’s fibrinogen level was normal, only the absence or dysfunction of the fibrinogen receptor could be the cause of the aggregation disorder. A flow-cytometric analysis of the surface of platelet membrane confirmed the defect of fibrinogen receptor, thus the diagnosis of Glanzmann thrombastenia was proven. The Glanzmann thrombastenia has three different types. In type I., due to the basically complete absence of GPIIb-IIIa there is no clot retraction; in type II the clot retraction is nearly normal since there are some fibrinogen receptors on the platelet surface. In the third, so called variant type the amount of GPIIb-IIIa is normal, however an abnormal receptor component is expressed on the surface. The molecular genetic and biochemical studies of a colleague of mine – Gergely Losonczy – showed that our patient belongs to the type I group.

The CTs of eight patients with Glanzmann thrombasthenia reported earlier was highly prolonged. The CT results suggest that PFA-100 is a valuable screening test for Glanzmann thrombasthenia, as well.

*Hermansky-Pudlak syndroma*

The data available in the literature on PFA-100 CT in storage pool disease represent only three publications. In two reports with a total of 5 patients the type of storage pool defect was not specified. In one of these patients only collagen/epinephrine CT was determined and significant prolongation was observed. In the remaining four patients no closure was observed with either of the cartridges. In a most recent publication six patients with HPS were investigated for PFA-100 CTs and all patients had abnormal CT with the collagen/epinephrine cartridge, but four values were only slightly above the reference interval. In the latter study only two patients had considerably prolonged CT with the collagen/ADP cartridge, the CT of three patients was slightly prolonged and one patient had normal CT.
In our study none of the HPS patients had prolonged CT with the collagen/ADP cartridge. There seems to be a logical explanation for this finding. HPS platelets lack dense granular ADP but the biochemical mechanism of platelet activation is usually intact. In the collagen/ADP cartridge the cartridge membrane is the source of ADP. Thus, no prolongation of CT is expected. With the collagen/epinephrine cartridge the CT was prolonged in four out of the five cases and in the remaining patient it was close to the upper limit of the reference interval but still in the normal range. At the same time all five patients had highly prolonged BT.

The partially contradicting results obtained in the case of Hermansky-Pudlak syndrome could be due to the facts that this disease is neither genetically nor phenotypically uniform.

Although the findings suggest that in detecting storage pool disease PFA-100 CT is not superior to template BT the data are too few to draw a firm conclusion.

Evaluation of the effect of acetylsalicylic acid (ASA) treatment on PFA-100 closure time

ASA irreversibly acetylates Ser 529 of the cyclooxygenase, resulting in inhibition of thromboxane A$_2$ (TXA$_2$) generation, thus inhibiting the formation of a molecule that plays central role in platelet activation and first of all in the mechanism of secretion.

The problem of ASA resistance and especially its diagnostic determination is still a heavily debated question without clear international guidance. According to our judgment most of the problems arise from the uncertainties caused by the variable definition of aspirin resistance. ASA resistance could be assessed by two different ways. Lack of acetylation of Ser 529 in the cyclooxygenase is called as chemical resistance. Failure of ASA therapy is the clinical (therapeutic) resistance. It is well known, that a group of
patients does not respond to ASA therapy even in the absence of chemical resistance. Initiating a new series of clinical tests with this group of patients the clinical investigators might have a chance to publish new and very likely interesting results. Another important question is the possibility of efficient ASA therapy in spite of the presence of chemical resistance. Although this possibility can not be completely excluded, there is no other excepted explanation for the therapeutic effectiveness of ASA than the inhibition of TXA$_2$ generation.

All laboratory methods used to evaluate the effectivity of ASA treatment can be applied to recognize only chemical resistance, clarification of the causative factor of therapeutic resistance can not be expected from these methods.

The widespread application of ASA as an antiplatelet agent – especially for preventing atherothrombotic events – asked for the introduction of such a screening test which quickly and adequately informs us about the compliance of the patients, about the chemical effectiveness of the drug and about the possible existence of chemical resistance.

The direct determination of the ASA (or salicylic acid) level in the plasma is not suitable for following the inhibitory effect of ASA on platelet function. These two compounds show quick pharmacokinetics, ASA can be detected in the circulation for 30 minutes after its administration while its effect on platelets keeps on for several days. Measuring the bleeding time is also inappropriate because of its insensitivity and its unsuitability for serial investigations. Aggregometric measurements without measuring secretion simultaneously could lead to false conclusions. The first phase of ADP aggregation is not secretion dependent, so the inhibition of TXA$_2$ synthesis does not influence it. It is not easy to find the ADP dose at which the primer and secondary aggregation could be evaluated separately. The epinephrine aggregation is either missing or strongly reduced even in some healthy individuals. The effect of ASA on collagen aggregation depends on the collagen concentration and this dependence
is widely variable in individuals. Among the aggregation studies the most unambiguous results are provided by the arachidonic acid aggregation. The simultaneous measurement of aggregation and ATP secretion (lumiaggregomeric measurement) makes the evaluation of the results much easier, however this a complex, time consuming and an expensive method which does not reflect precisely the complex in vivo circumstances and it can be conducted only in a laboratory. Measurement of the PFA-100 closure time is a quick technique, it could be executed at the bedside in a hospital and this method closely approximates the in vivo circumstances.

Our findings suggest that the PFA-100 equipment is suitable for detecting ASA effect. Undoubtful that usage of the PFA-100 equipment occupies an illustrious position among the laboratory methods applied for this purpose, it is internationally recommended. We can not say, however that with the usage of PFA-100 the determination of ASA resistance is solved, since in the case of elevated vWF ristocetin cofactor activity prolongation of the closure time could not take place when arachidonic acid aggregation is used to prove ASA effect. In any case, it would be necessary to have a 100% sure – mass spectrometric or even a complicated isotope labeling – reference method whose comparison with other clinical methods using a large number of patients could help to choose the most effective laboratory method.

Diagnostic value of PFA-100 closure time measurements for detecting impaired platelet function during thrombolytic therapy

Thrombolytic therapy is a major therapeutical approach in treating acute myocardial infarction (MI). Early administration of thrombolytic agents results in lower mortality and preservation of ventricular function. However, thrombolysis by different plasminogen activators has its limitations. Complete reperfusion is achieved only in about 50% of the patients, and 10-15 % of initially recanalized coronary vessels reocclude. Bleeding, even with modern
thrombolytic agents, remains a concern and intracranial hemorrhage occurs in 0.3-1.5% of the patients. Both bleeding complications and reocclusion have been associated with platelet dysfunction. Depending on experimental or clinical conditions, plasmin generation can both activate and inhibit platelets and initial platelet activation might be followed by the development of impaired platelet function. To prevent platelet activation and, by this way, reocclusion the thrombolytic therapy is regularly supplemented with ASA.

Since the introduction of thrombolytic therapy there has been efforts to find appropriate laboratory test(s) that would predict the bleeding risk of patients undergoing such therapy. Unfortunately, clotting tests used for the detection of “lytic” state proved to be unsatisfactory in this respect. So far, standardized bleeding time, a screening test for impaired platelet function, had the best performance showing 69% sensitivity and 69% specificity in predicting spontaneous bleeding during thrombolysis. Bleeding time carried out with a disposable device is quick, easy to perform, however, it is difficult to standardize, its predictive value is still only 43% and it is hardly applicable for serial investigations during thrombolysis. No laboratory test of platelet activation is used for the prediction of reocclusion, although ADP-induced platelet aggregation has been proposed for such purpose. Prolongation of CADP closure time before the initiation of thrombolytic therapy indicates impaired platelet function that might represent an increased risk for bleeding during thrombolysis. In one third of the patients with normal CADP closure time before the initiation of thrombolytic therapy a significant (up-to 97 sec) prolongation of this closure time occurred during thrombolysis. As ASA is without any effect on CADP closure time its prolongation during thrombolytic therapy indicates that mechanism(s) other than the inhibition of the cyclooxygenase pathway are involved in the development of impaired platelet function. In addition to being useful in screening for inherited platelet disorders, PFA-100 seems to be a valuable tool in detecting acquired platelet defects, as
well. By using the ASA-insensitive CADP cartridge deterioration of the haemostatic function of platelets by thrombolytic therapy can be monitored even during ASA treatment.

A further advantage of measuring PFA-100 closure times of MI patients undergoing thrombolytic therapy is the verification of the effectiveness of ASA treatment. CEPI closure time measured before the start of thrombolysis provides information on the effectiveness of previous ASA therapy. The absence of prolongation of CEPI closure time would indicate non-responsiveness or non-compliance. The effectiveness of ASA administered as part of the thrombolytic therapeutic regimen could also be controlled by CEPI closure time measurement. Although the effect of fibrinolytic therapy on platelets could contribute to the prolongation of CEPI closure times, the lack of prolongation clearly indicates the ineffectivity of ASA therapy. The finding that 7 out of 26 patients could be considered as non-responders underlines the importance of such measurement.

It is interesting that in three patients being on long-term ASA treatment prior to the onset of MI the originally highly prolonged (>300 sec) CEPI closure time significantly decreased within 2 hours after the introduction of thrombolysis.

PFA-100 was designed to detect platelet defects and not platelet activation. For this reason the membranes of the cartridges are impregnated with combinations of platelet agonists and the relatively short closure times corresponding to the reference interval make it difficult to detect accelerated closure of the aperture. There is only a single, most recent report in which shortened closure times in MI patients were attributed to in vivo platelet activation or elevated sensitivity of platelets to activating agents. When the closure time is highly prolonged, like the CEPI closure time in patients responding to ASA, its shortening could be a more sensitive indicator of in vivo activated platelets.
It is now well established that thrombolytic therapy as well as plasmin has a dual effect on platelets. The actual response depends on the duration of treatment, the actual plasmin concentration and individual variations also play a major role. The inhibition of platelet aggregation by fibrin(ogen) degradation products and the truncation of thrombin receptor by plasmin seem to be the primary causes of functional platelet defect. Degradation of vWF might also be involved, the proteolysis of its adhesive receptor (glycoprotein Ib-IX), however, does not seem to be a major contributor. On the other hand, plasmin and plasminogen activators have been shown to cause platelet aggregation, secretion, an increase in cytosolic Ca^{2+}, inositol triphosphate synthesis and activation of protein kinase C. They also potentiate platelet responses to low doses of a number of aggregating agents. Plasmin is capable of cleaving and activating thrombin receptor at the same site as thrombin and by limited proteolysis of the amino-terminal domain of platelet membrane glycoprotein IIb it can irreversibly transform glycoprotein IIb and IIIa complex into an active fibrinogen receptor. The overall effect of these complex mechanisms, depending on individual responsiveness, may manifest in impaired platelet function leading to bleeding tendency or in enhanced platelet activity contributing to the reocclusion process. Monitoring of platelet function during thrombolytic therapy may provide important information on the actual responsiveness of platelets. The present study suggests that measuring PFA-100 closure times could be a reliable tool for detecting impaired platelet function during thrombolytic therapy. It remains to be seen if prolongation of CADP closure time can be correlated to bleeding episodes or if shortening of CEPI closure time in ASA treated patients indicates in vivo platelet activation and an increased risk of reocclusion. The results suggest that measurement of PFA-100 closure time is a useful tool for monitoring platelet function during thrombolytic therapy. It also provides information on the effectiveness of ASA administered before or during thrombolysis.
To summarize, the published data and the results of our work suggest that the PFA-100 Platelet Function Analyzer with the joint application of collagen/ADP and collagen/epinephrine cartridges is suitable for detecting congenital and acquired platelet functional disorders. It might be used as a screening assay or as a test complementary to traditional platelet function assays.

Publications on which this thesis is based on:


5. Kerényi A, Soltész P, Veres K, Szegedi Gy, Muszbek L. Monitoring platelet function by PFA-100 closure time measurements during
thrombolytic therapy of patients with myocardial infarction. Thromb Haemost 2005;116:139-44. IF: 1.541


Publications not used in the thesis


