The effects of platelet receptor GPIIb/IIIa polymorphism (Leu Pro33) on the receptor expression and platelet aggregation in patients with ischaemic stroke

Endre Pongrácz1, Katalin Schweitzer2, József Fürész2, János Fent2, Attila Tordai3, Zoltán Nagy4

1Department of Neurology, Stroke Unit, Central Hospital of Home Office, Budapest, Hungary
✉ pongracze@gmail.com
2Department of Pathophysiology, Institute of Health Protection, Hungarian Defence Forces, Budapest, Hungary
3Department of Molecular Genetics, National Institute of Haematology, Budapest, Hungary
4Department of Vascular Neurology, National Stroke Centre, Semmelweis University, Budapest, Hungary

Received: Aug 26, 2006 • Accepted: Sep 6, 2007

ABSTRACT

Platelet hyperaggregation in ischaemic stroke patients is a proven finding, and associated with increased expression of the platelet surface GPIIb/IIIa receptor. The polymorphism occurs at nucleotide position 1565 of the GPIIIa gene resulting a 33Leu-Pro change. Data are conflicting regarding the abnormal function of the PlA1/A2 receptor in stroke. The aim of the study was to address the difference of platelet receptor function in ischemic stroke patients with the wild PlA1/A1 and heterozygous PlA1/A2 genotype.

A total of 51 patients with PlA1/A1 and 54 patients with PlA1/A2 genotypes were enrolled. Polymerase chain reaction was used for genotyping of platelets. Platelet aggregation was measured in whole blood and in platelet rich plasma (PRP). Flow cytometry was used for measuring surface molecule expression (CD42b, CD41a, CD61, CD62P) and fibrinogen binding capacity of cells with phosphate buffer solution (PBS) in comparison with activation by ristocetin in whole blood as well as by adenosine diphosphate (ADP) in PRP.

In comparison with wild types, platelets carrying the PlA1/A2 genotypes showed hyperaggregation measured in whole blood and induced by ristocetin (p< 0.05). Using whole blood flow cytometry with ristocetin induction, the CD62P+/FIB- (P selectin) and the CD62P+/FIB+ were more expressed in heterozygous platelets as compared to wild types (p< 0.01 and p< 0.05), respectively. According to mean fluorescence intensity with ADP induction, an increased expression of CD61*, CD61*/CD41* and CD62P* in PlA1/A2 platelets were detected as compared to the group carrying the wild type (p< 0.0001, p= 0.006, p= 0.0001), respectively.
INTRODUCTION
Disturbances in primary hemostasis, particularly platelet aggregation, play a significant role in the pathogenesis of obliterative arterial diseases. In the intracranial arteries supplying the central nervous system, thrombi are usually formed at branches characterized with high shear flow (i.e. bifurcation of the carotid arteries, syphon of the arteriae cerebri mediae and circulus arteriosus Willis) and mainly consist of platelets and fibrin (white thrombus). Venous thrombi are formed predominantly under low shear conditions (i.e. venous valves, cerebral sinusoids) and characterized by little fibrin formation with many red blood cells (red thrombus). Central nervous system damages of vascular origin are mediated partly by alterations inducing hemostatic cascade mechanisms, ending in prothrombotic conditions. This process includes facilitation of platelet adhesion and aggregation, endothelial dysfunction, structural endothelial damage and thrombophilic alteration of plasma protein[1-3]. Damage to the endothelium leads to exposition of the highly thrombogenic components of the subendothelium, particularly collagen. Platelets become tethered to the collagen, through the glycoprotein (GP) surface receptor GPIb-von Willebrand factor (vWF) interaction, which is reversible. Platelet adhesion is mediated via GPVI and GPIa/IIa, while the GPVI is the primary receptor underlying platelet activation and leading to aggregation through GPIIb/IIIa, which is one of the most important platelet surface receptors. In this process, vWF is bound and platelets are adhered to the endothelium. This event is followed by GPIb-vWF binding, which activates the GPIlb/IIa (CD41/61) receptor that binds to vWF resulting in reversible platelet aggregation[4,5]. During the aggregation process, platelets release a number of aggregating factors including adenosine diphosphate (ADP), serotonin, etc. Binding of ADP to purine receptors induces further change in platelet function. GPIIb/IIIa-fibrinogen binding is formed, making the aggregation irreversible[6]. Some years ago, a genetic polymorphism affecting the platelet GPIIb/IIIa receptor (Leu-Pro33) at position 1565 was described[7]. In the present paper, we address the question if the in vitro function of P1A1/A1 (wild type) platelets are different from P1A1/A2 in ischemic stroke patients. In vivo activation processes were modelled by ex vivo studies.

MATERIALS and METHODS
Patient Selection
A total of 105 consecutively patients were enrolled in ischemic stroke of origin. Patient group having P1 A1/A1 genotype (as controls) consisted of 51 patients diagnosed with computerized tomography (CT), magnetic resonance imaging (MRI), and echocardiography (ECHO). Blood samples were collected for analysis of platelet function after two months of onset of stroke. Patients were in the chronic stage of ischemic stroke disease.

Patient group having P1 A1/A2 genotype (as verum group) consisted of 54 patients with CT or MRI diagnosed. Risk factor definition were the following: hypertension (blood pressure > 140/90 mmHg), hyperlipidaemia (serum cholesterol > 5.2 mmol/L, triglyceride > 2.2 mmol/L), smoking (over 10 cigarettes/day), alcohol consumption (more than 40 g/d concentrated alcohol, respectively). Antiplatelet therapy was discontinued 14 days before the study. Exclusions criteria were: stroke patients with hemorrhage intracerebral sinus...
thrombosis or subarachnoideal hemorrhage, as well as individuals taking other drugs influencing platelet function [e.g. nonsteroidal anti-inflammatory drugs (NSAIDs)]. All participants were fully informed about genetic tests and gave their written consent. The study was approved by the Regional Research Ethic Commission. Baseline characteristics of study patients presented in Table 1.

Platelet aggregation and adenosine triphosphate (ATP) release were assessed in both groups. Ristocetin was used as inductor in order to inform us about the functionality of vWF receptors via GPIb-vWF and GPIIb/IIIa-vWF binding. Collagen, which is released after endothelial damage and induces secondary aggregation in vitro by inducing platelet release, was also used in the study. In addition, activation by ADP was tested, which is known to induce primary and secondary aggregation of the irreversible type.

Based on the data of our whole blood aggregation studies, we performed flow cytometry assays with ristocetin stimulation in the second part of our study. Moreover, ADP was used for flow cytometric studies in platelet rich plasma (PRP), with the use of more induc- tors (thrombin, ADP, and collagen); irreversible aggregation was performed after ADP release. In parallel specimens, with and without activation, flow cytometry analyses of CD42b (GPIb), CD41a (GPIIb/IIIa complex), CD61 (GPIIia) and CD62P (P selectin) surface markers and bound fibrinogen were performed. Since GPIIia is expressed on megacyocytes, some B-lymphocytes, leukemic cells and non-haematopoietic cells, no CD61 antibodies were applied in the whole blood studies. Only CD41a (GPIIb/IIIa complex) was assayed in whole blood. In PRP, CD41/CD42b, CD41/CD61 as well as CD62P were studied, the latter being the central issue of our investigations.

1. Method of genetic polymorphism detection: Genomic DNA was extracted from peripheral blood samples using standardized techniques. In order to detect GPIIb/IIIa polymorphism, samples were screened for the presence of an MSP restriction site (CCGG), which only exists on the P1A2 allele as was published by Ying et al.[8]. Two of the P1A1/A1 and two of the P1A1/A2 patients were investigated on the same day.

<table>
<thead>
<tr>
<th>Table 1. Baseline characteristics of study patients.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient group</strong></td>
</tr>
<tr>
<td>$PL_{A1/A1}$</td>
</tr>
<tr>
<td>n= 51 (%)</td>
</tr>
<tr>
<td>Gender</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Age/(years ±)</td>
</tr>
<tr>
<td>Personal risk profile</td>
</tr>
<tr>
<td>Hypertension</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>Cigarette smoking</td>
</tr>
<tr>
<td>Alcohol consumption</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
</tr>
<tr>
<td>Pathomechanism of ischemic stroke</td>
</tr>
<tr>
<td>- Large vessel wall stenosis &gt; 50%</td>
</tr>
<tr>
<td>- Lacunar infarction</td>
</tr>
<tr>
<td>- Cardiogen embolism</td>
</tr>
<tr>
<td>- Lacunar infarction with leukoaraiosis</td>
</tr>
<tr>
<td>- Undetermined of origin</td>
</tr>
</tbody>
</table>
2. Assessment of platelet function: Blood was taken from the antecubital vein under aseptic conditions with Becton Dickinson vacutainer into a tube containing 3.8% sodium citrate. Platelet aggregation in whole blood and in PRP and flow cytometric analyses were performed.

2.1. Platelet aggregation was measured using the Ingerman-Wojenski procedure\[9\] in whole blood using a probe assembly inserted into a blood-containing cuvette. Changes in electrical resistance were measured and quantified in ohms. Platelet aggregation was measured in presence of luciferin-luciferase, following the induction with three different mediators, namely: ADP 5 µM, collagen 5 µg/mL, and ristocetin 1.25 mg/mL final concentrations, all of them Chrono-Par (Chrono-Log) The name of the reagent was Chrono-Lume No.395 luciferase-luciferin, corporation: Chrono-Log.

2.2. Platelet aggregation in PRP was measured with the procedure described by Born\[10\] using Chrono-log 560 whole-blood lumi-aggregometer (Chrono-log Corp. USA) with ADP 5 µM (Chrono-log), collagen 5 µg/mL (Chrono-log) and ristocetin 1.25 mg/mL (Chrono-log) as inductors, in a final concentration. We detected optical density changes of the platelet suspension. Results were given in percents, with 100% defined as the optical density of PRP 0% as the one of platelet poor plasma. Separation of PRP was standardized using a programmable centrifuge (Hereaus Sepatech Omnifuga 2.0 RS) at a setting of 200 g and 10 minutes without cooling and deceleration.

2.3. Release of ATP was detected by the luminescence generated by buffered firefly extract, which has been added to the sample and becomes luminescent in the presence of ATP. The aggregation and secretion simultaneously reflect in the synthesis of biologically active prostaglandine intermediates.

Flow cytometry assay of platelet function: Flow cytometry assay was conducted in whole blood according to the Michelson procedure\[11\]. Whole blood was diluted 1:9 with phosphate buffer solution (PBS). In all specimens, anti-CD41a/anti-CD42b, anti-CD62P/anti-fibrinogen and anti-CD41a/anti-CD62P dual-colour labelled were assessed on 5000 platelets without or with ristocetin 0.62 mg/mL final concentration for preactivation. The fluorescence signals of the unstained control and isotypic control were also determined on each sample. Platelets were identified by light scatter characteristic and the binding of anti-CD41. The anti-CD42b, anti-CD62P binding and fibrinogen binding were analysed by EpicsElite (Coulter). Flow cytometry in PRP was performed as described by McGregor et al.\[12\], PRP was diluted (1:9) in PBS, incubated for 10 minutes with ADP (2.5 µM final concentration) or PBS buffer and with as follow as antibodies in darkness at room temperature. The platelets were dual-color labelled by anti-CD61 FITC (Sigma)/anti-CD41 PE (Immunotech) and anti-CD41 FITC (Immunotech)/anti-CD42b PE (Immunotech) and anti-CD62 PE (Sero-tech) antibodies. Appropriate isotypic control reagents were used. These specimens were analysed by a FACScan (Becton Dickinson) device, 5000 platelets per sample were collected. The mean fluorescence intensity and percent of cells expressing the special antibody were given. The device was calibrated with “CaliBrite” (Becton Dickinson) fluorescent microbeads.

Statistical Analysis
Mann-Whitney U test was used for comparisons of groups. A value of p< 0.05 was regarded as significant.

RESULTS
Platelet Aggregation Studies
Aggregation was induced by ADP, collagen and ristocetin in whole blood, in presence of luciferin-luciferase. Platelets having P1A1/A2 genotype showed an increased aggregation with each inductor as compared to the wild type. In cases of ristocetin induction the difference was significant (p< 0.05). In this study, ATP release was also measured (P1A1/A1 0.18 nM ATP, P1A1/A2 0.54 nM ATP on ristocetin sample), and no significant difference was observed between the two groups.
Measuring in PRP, platelet count was 285 ± 41 in the P1A1/A1 group and 244 ± 84 in the P1A1/A2 group. The level of aggregation induced by ADP, collagen or ristocetin was > 85%. There was no difference between the two groups (not shown).

**Flow Cytometry Assay in Whole Blood**

The CD42b+ expression in whole blood (not shown) on resting platelets differed the P1A1/A1 and P1A1/A2 in CD42b+ activity, but the difference did not reach a level of significance. In specimens preactivated with ristocetin, CD42b+ activity decreased in both groups but significant difference was not detected.

The percent of CD62P+ positivity in resting platelets was nearly equal in both groups.

After ristocetin induction, the percentage of CD62P in cells having P1A1/A1 increased from 5% to 9% (n.s.). In the heterozygous group, the CD62P positive cells increased significantly from 4% to 14% (p< 0.005) (Figure 2/A).

The CD62P/fibrinogen dual positive cells were also assessed as shown in Figure 2/B. There was no significant difference between the two groups in resting platelets incubated with PBS. The P1A1/A1 group showed no increase in the expression following ristocetin activation, but the expression was detected with significant increase in the P1A1/A2 group.

**Flow Cytometry Assay of PRP**

In PBS-incubated specimens, there was not a significant difference on the percentage of CD41/CD42b dual positive platelets on the wilde and mutant groups. Following ADP activation, the percentage of dual positive cells decreased to some degree as compared to the platelets of resting state (Table 2).
CD 42b$^+$ expression was assessed by detecting the fluorescence intensity of the same platelets (Figure 3/A), but no significant differences were found between the two groups.

As Table 2 shows, the proportion of CD61/CD41 positive cells following PBS incubation and ADP induction were equal in the two groups. The same platelets incubated by PBS showed equal fluorescence intensity of CD61/CD41 in both groups (Figure 3/B), while a very significant increase was detected after ADP induction in both groups, favoring the $\text{P}_{\text{A1/A2}}$ group ($p=0.006$).

Figure 3/C shows the fluorescence intensity of CD61$^+$ in resting platelets, without significant group difference. Following ADP induction, an increase in CD61$^+$ fluorescence intensity was noted in both groups, favouring the heterozygous group ($p<0.0001$).

According to Table 2, no differences were detected between the two groups in percent of CD62P$^+$ in PBS-incubated specimens. After ADP induction, the detected expression of CD62P$^+$ was increased up to 48% on the platelets surface having the wild type and up to 56% in carriers of $\text{P}_{\text{A1/A2}}$. According to the data of Figure 3/D, the fluorescence intensity of CD62P$^+$ platelets after PBS incubation did not differ significantly between the two groups. Following ADP induction, both groups showed a significant increase in CD62P$^+$ expression, which was higher in the $\text{P}_{\text{A1/A2}}$ group ($p=0.0001$).

**DISCUSSION**

Earlier findings suggest an association of the platelet receptor GPIIb/IIIa polymorphism with unexpected myocardial infarction and stroke in apparently healthy individuals[13,14].
Furthermore, the polymorphism is supposed to be linked to a prothrombotic state\[15\]. In other publications, the P1A2 allele was not found to be associated with an increased risk of myocardial infarction or ischemic stroke in young patients\[16,17\]. On the other hand, Feng\[18\] has found a hyperaggregability of heterozygous platelets induced by epinephrine in a large cohort of healthy persons. The investigator hasn’t used any other methods beyond aggregometry. The exact mechanism of the conformation changing of the GP IIIa part of the receptor is not clear.

The prevalence of this polymorphism (P1A1/A2) is 16-19% in the healthy population of Germany\[19,20\] and 15% in Austria\[21\]. A different in prevalence was found in the United States: 16% in African-Americans 20% in the Caucasian population\[22\]. According to our previous study, the prevalence of P1A1/A2 with P1A2/A2 genotypes was 23% in healthy subjects (n= 173) and it was found to be 30.2% in ischemic stroke patients (n= 253)\[23\]. These data suggest that the P1A1/A2 polymorphism is common in the Caucasian population.

In the presented study, we found that platelets carrying P1A1/A2 genotypes of GPIIIa receptors show hyperaggregation as measured in whole blood in presence of ristocetin. Using whole blood flow cytometry, the CD62P+ / FIB- (P selectin) and CD62+/FIB+ were more expressed in heterozygous platelets as compared to those with the wild type. In another part of this study, detection of mean fluorescence intensity after ADP induction revealed a significant increase in CD61+, CD61+/CD41+ and CD62P+ of P1A1/A2 platelet receptor expression as compared to the group carrying the receptors with wild type.

The ATP release was increased in the P1A1/P1A2 group compared to the group having wild genotype; however, this value was not significant.

Few and conflicting data are available in the literature regarding the in vitro properties of platelets carrying the GPIIb/IIIa (LeuPro33) genetic polymorphism (Table 3).

There are some investigators who have found a hyperaggregability of platelets in subjects carrying this polymorphism, leading to prothrombotic condition in cardio- and cerebrovascular diseases of ischemic type\[18,24,25\]. However, other authors did not find any differences in ADP induction and fibrinogen binding capacity of platelet surface receptor functions in platelets having the P1A1/A1 and P1A1/A2 genotypes\[26-28\]. Our goal was to clarify this issue by performing platelet aggregation and flow cytometry studies in a complex setup. For this purpose, we performed analyses in whole blood as well as in PRP. We found that in whole blood, after using ristocetin induction, platelets with P1A1/A2 show increased affinity to aggregate as compared to platelets with wild types (Figure 1). This finding underlines the importance of the vWF receptor functionality. Measurements in PRP indicated 80-90% of platelet aggregation without group difference. The aggregation procedure, considered as the gold standard, seems to be less suitable for the detection of hyper-aggregability\[29-31\]. We investigated the expression of the GPIIa surface receptors (CD61). Two experimental settings—one in whole blood and one in PRP—can provide more accurate answers to this question. The study with whole blood flow cytometry indicated that GPIIb/IIIa expression is more pronounced in P1A1/A2 platelets than in P1A1/A1 ones, and the fibrinogen binding capacity after activation was higher in the heterozygous group. A specialized flow cytometry assessment in PRP revealed that the GPIIa receptor is expressed in 80-90% on the surface of resting cells. After activation, this proportion did not increase (Table 2). In contrast to these findings, fluorescence intensity showed that the number of GPIIb/IIIa receptors per platelet increased after ADP activation, and it was highly significant in P1A1/A2 platelets. One may conclude that this polymorphism of the gene encoding the platelet surface receptor GPIIb/IIIa can lead to pathologic changes in receptor function. A
lower threshold of alpha-granule release can be part of the alteration as it was described by Michelson et al. [29]. Based on the results of this study, platelets carrying P1A1/A2 genotypes differ from the wild type in several aspects of their receptor function: first, their receptors are expressed in more copies per platelet following ADP activation (Figure 2); second, this expression can result in higher affinity for fibrinogen (Figure 2), as well as in hypersensitivity of vWF receptors (Figures 1 and 2). Our findings support the notion that hyperaggregability of P1A1/A2 platelets may occur as a result of several mechanisms and, due to its permanent nature, may lead to increased thrombophilic potential.

### Acknowledgements

The authors wish to express their gratitude to Dr. Ede Frecska (National Institute of Psychiatry and Neurology, Budapest, Hungary) for his help in the preparation of the manuscript and to Mrs. Eszter Lengyel for her technical assessment.

### REFERENCES

The effects of platelet receptor GPIIb/IIIa polymorphism (Leu Pro33) on the receptor expression and platelet aggregation in patients with ischaemic stroke


