Increased binding of fibrinogen to glycoprotein IIla-Proline33 (HPA-1b, PlA2, Zw b) positive platelets in patients with cardiovascular disease

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Aims The GPIIb-IIIa complex on the platelet membrane plays an important part in thrombosis as it is the receptor for fibrinogen. The gene for platelet membrane glycoprotein IIIa has multiple alleles one of which, the GPIIIa-Proline33 (HPA-1b, PlA2, Zw b) allele has been reported in some, but not all studies, to be associated with an increased risk of myocardial infarction. We investigated whether the presence of the Pro33 form of GPIIIa on the platelet membrane is associated with increased fibrinogen binding.

Methods and Results Blood samples from 70 patients (54 male) with stable angina of whom 22 (18 male) had a history of previous myocardial infarction, were analysed for the GPIIIa-Leu-Pro33 polymorphism at the genomic level, and for whole blood flow cytometric measurement of platelet fibrinogen binding. The GPIIIa-Pro33 form was present in 20 (28.6%) patients (1 homozygous) representing an allele frequency of 0.85 and 0.15 (GPIIIa-Leu33:Pro33). The incidence of myocardial infarction was higher (40.0%) in patients positive for GPIIIa-Pro33 than in those without (32.0%) but this was not significant (P=0.58).

Fibrinogen binding to ADP-stimulated platelets was significantly higher in the GPIIIa-Pro33 positive group at all ADP concentrations (<0.0001; two way ANOVA). There was no association between fibrinogen binding and the level of expression of the GPIIb-IIIa complex, platelet volume or platelet count. Fibrinogen binding in response to thrombin stimulation was not different between the groups (P>0.05).

Conclusions The increased tendency of platelets from patients with the Pro33 form of GPIIIa may predispose patients with this allele to a higher risk of acute thrombotic events, and argues for selective use of therapeutic agents that inhibit ADP-mediated platelet activation in occlusive vascular disease states.

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Key Words: Platelets, glycoprotein IIb-IIIa, polymorphism, fibrinogen, flow cytometry.

See page 706 for the Editorial comment on this article

Introduction

Platelets play an important role in acute thrombotic events, including myocardial infarction[1]. This is supported by the efficacy of antiplatelet therapy in preventing and reducing mortality and morbidity in large randomized trials[2,3]. Platelet hyper-reactivity has also been shown to be associated with unstable coronary syndrome[4] and with a poor outcome in patients following myocardial infarction[5] or angioplasty[6].

Thrombus formation is dependent on fibrinogen binding to the platelet membrane, glycoprotein (GP) IIb-IIIa complex[7]. Trials of fibrinogen receptor antagonists have demonstrated the importance of this interaction in vivo in preventing thrombosis in unstable angina[8], in high risk coronary angioplasty[9] and in acute myocardial infarction treated with thrombolysis[10]. GPIIb-IIIa is a transmembrane heterodimer and a member of the β3 integrin family. The GPIIb and GPIIIa glycoproteins are encoded by separate, but closely associated genes on the long arm of chromosome 17[11]. Both genes have
allelic variants based on single nucleotide changes leading to single amino acid substitutions. One of these, the GPIIIa-Leu-Pro33 variant (also known as the bi-allelic HPA-1, PlA or Zw system), has, in the Caucasian population, frequencies of 0.85 and 0.15 for the Leu33 and Pro33 encoding alleles, respectively. Recently Weiss et al. reported that positivity for the PlA2 (GPIIIa-Pro33) allele was associated with a 2.1 times increased risk of myocardial infarction in a group of 71 patients from the Baltimore area; this increased risk was even greater (3.6 times) in patients who had a myocardial infarction under the age of 60. This original observation, together with an anecdotal report of a sudden cardiovascular death in an Olympic athlete who was homozygous for the GPIIIa-Pro33 encoding allele, led us to test whether platelets from GPIIIa-Pro33 positive individuals have an increased tendency to bind fibrinogen. Fibrinogen binding to platelet GPIIb-IIIa was measured using a whole blood, flow cytometric technique in blood from 70 patients with angiographically confirmed stable angina.

**Methods**

**Subjects and blood sampling**

A total of 70 subjects listed for elective coronary angioplasty were recruited. All had stable angina pectoris and significant stenosis in one or more of their coronary arteries (defined as a reduction of greater than 50% of the luminal diameter). The majority of patients (64/70) were European Caucasians, with the remaining six of Indian Asian origin. Anti-anginal medication was standardized for all patients to a beta-blocker and aspirin only prior to testing. The study was approved by the Ethical Practices Committee of the Royal Brompton Hospital.

Genomic DNA was extracted from EDTA anticoagulated blood samples and citrate anticoagulated blood samples were collected for flow cytometric analysis of platelet membrane antigen expression. DNA analysis

Genomic DNA was analysed for the T-C mutation at base 196 in exon 2 of the GPIIIa gene by the polymerase chain reaction using two sequence-specific primers amplifying either the GPIIIa-Leu33 or GPIIIa-Pro33 coding alleles as described previously. Each reaction was controlled by the inclusion of a pair of primers which amplified a 429 base-pair fragment of human growth hormone.

**Platelet studies**

Platelet bound fibrinogen was detected with a FITC-conjugated, polyclonal, rabbit antibody to human fibrinogen (Dako Ltd, High Wycombe, Bucks, U.K.). Platelet membrane GPIIb-IIIa and GPIbα expression were measured with FITC labelled murine monoclonal antibodies; RFGP56 (CD41) and RFGP37 (CD42b), respectively. The negative control consisted of FITC-conjugated, non-immune, mouse IgG (Coulter Immunology, Luton, Beds, U.K.). Blood samples were prepared for flow cytometric analysis by a standardized, whole blood method, as described previously. Samples were analysed either without stimulation, or in the presence of ADP 0.01–10 μmol.l⁻¹ or human α-thrombin 0.02–0.32 units.ml⁻¹ (Sigma Chemical Co Ltd, Poole, Dorset, U.K.). Results were recorded as either the percentage of fluorescence-positive particles (fibrinogen binding) or as the mean fluorescence intensity (GPIIb-IIIa and GPIbα expression).

Mean platelet volume was measured in blood anticoagulated with EDTA and stored for 2 h prior to analysis.

**Statistical analysis**

All data are given as mean ± SD or SEM. Differences between platelet variables were calculated using either two-way ANOVA or Student’s two-tailed t-test, as appropriate, and differences between patients’ variables were calculated using Fisher’s exact test, all using GraphPad InStat software. Relationships between independent variables were calculated by Pearson’s correlation using Excel 5.0 software.

**Results**

**Patients’ characteristics and DNA analysis**

Of the 70 patients 16 were female and 54 male and 22 had a myocardial infarction previously (4 females and 18 males). The female patients were significantly older than the males (63.4 ± 9.4 against 58.0 ± 9.4; mean ± SD; P=0.048).

The distribution of the GPIIIa genotypes in the patients is shown in Table 1. Of the 70 patients, 50 were homozygous for GPIIIa-Leu33, 19 heterozygous and one homozygous for GPIIIa-Pro33. The observed allele frequencies of 0.85 and 0.15 are in line with those previously reported for Caucasian populations.
Comparison of the mean ages of the 50 GPIIIa-Pro33−ve and 20 GPIIIa-Pro33+ve patients showed no difference between the groups (Table 2). The incidence of myocardial infarction was higher in the GPIIIa-Pro33 positive patients than in the negative patients (40.0% against 32.0%) but this was not significant (odds ratio of 0.71; 95% CI 0.24–2.07; *P*=0.58). There were no differences between the groups as regards a history of smoking, hypertension, diabetes or hypercholesterolaemia (Table 2). All patients had normal levels of plasma fibrinogen.

Platelet fibrinogen binding and glycoprotein expression

Fibrinogen binding to GPIIb-IIIa on unstimulated platelets was not different in the two groups (1.44±0.93% +ve for GPIIIa-Pro33−ve compared to 1.08±0.72% +ve for GPIIIa-Pro33+ve; *P*=0.125). However, GPIIIa-Pro33+ve platelets bound significantly higher amounts of fibrinogen when stimulated with ADP, when compared with GPIIIa-Pro33−ve platelets (Fig. 1; *P*<0.0001; two-way ANOVA). No difference in fibrinogen was seen between the groups following thrombin stimulation (Fig. 2; *P*>0.05; two-way ANOVA). Whilst not statistically significant it is of interest that platelets from the one patient homozygous for the Pro33 form of GPIIIa were even more responsive to ADP stimulation than the cohort of heterozygous patients (indicated in Fig. 1).

As shown in Fig. 3, total expression of the GPIIb-IIIa complex, measured as mean fluorescence intensity per platelet, was higher in the GPIIIa-Pro33 +ve patients (15.4±2.4 compared to 14.3±2.6), as was the level of expression of GP Ibα (15.1±2.5 compared to 14.1±2.3), but neither was statistically significant (*P*>0.1). Fibrinogen binding did not correlate with levels of GPIIb-IIIa (r<0.3; *P*>0.1 for all). The mean platelet volume was not significantly different between the two groups (9.24±0.88 fl for GPIIIa-Pro33+ve and 9.34±1.13 fl for GPIIIa-Pro33−ve) and there was no correlation between mean platelet volume and expression of either GPIIb-IIIa or GP Ibα, or fibrinogen binding; nor were there correlations with platelet count or plasma fibrinogen levels (*P*>0.2 for all). There was no association by subgroup analysis between these differences and a history of smoking, hypertension, diabetes or hypercholesterolaemia. Analysis of these data in relation to another known polymorphic site on GPIIb-IIIa, the Ile843Ser polymorphism on GPIIb responsible for the HPA-3/Bak alloantigen, showed no relationship between fibrinogen binding and genotype (data not shown).

Table 2 Association of relative risk factors with the PlA2 allele

<table>
<thead>
<tr>
<th></th>
<th>GPIIIa-Pro33 negative</th>
<th>GPIIIa-Pro33 positive</th>
<th>Relative risk (odds ratio ± 95% CI)</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>50</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>59.8 ± 8.5</td>
<td>58.2 ± 10.3</td>
<td></td>
<td>0.51</td>
</tr>
<tr>
<td>Previous MI</td>
<td>16 (32.0%)</td>
<td>8 (40.0%)</td>
<td>0.71 (0.24–2.07)</td>
<td>0.58</td>
</tr>
<tr>
<td>Females/male</td>
<td>9/41 (18%)</td>
<td>7/12 (35.0%)</td>
<td>0.51 (0.17–1.57)</td>
<td>0.25</td>
</tr>
<tr>
<td>Smoking history</td>
<td>5/42 (10.0%)</td>
<td>1/15 (6.3%)</td>
<td>1.79 (0.19–16.6)</td>
<td>1.00</td>
</tr>
<tr>
<td>Hypertension</td>
<td>13/34 (37.5%)</td>
<td>6/10 (37.5%)</td>
<td>0.64 (0.19–2.11)</td>
<td>0.53</td>
</tr>
<tr>
<td>Diabetes</td>
<td>3/44 (6.4%)</td>
<td>2/14 (12.5%)</td>
<td>0.48 (0.07–3.15)</td>
<td>0.59</td>
</tr>
<tr>
<td>Cholesterol ≥5.5 mmol . l⁻¹</td>
<td>1/46 (2.1%)</td>
<td>1/15 (6.3%)</td>
<td>0.33 (0.02–5.54)</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Figure 1 The percentage of platelets with bound fibrinogen in samples of blood stimulated with ADP. (●=GPIIIa-Pro33−ve/PlA1, n=50; ○=GPIIIa-Pro33+ve/PlA2, n=20; □=GPIIIa-Pro +ve/PlA2 homozygous patient, n=1). Data are presented as mean ± SEM.

Discussion

This study demonstrates an increased capacity for fibrinogen binding to the GPIIIa-IIIa-Pro33 variant. This was not seen in unstimulated, or thrombin-stimulated platelets but occurred when platelets were stimulated with ADP. The higher levels of fibrinogen binding in the GPIIIa-Pro33+ve group were not the result of greater receptor density, nor were these
differences related to platelet size or platelet count as there was no statistical correlation with fibrinogen binding or receptor expression (r<0.4; P>0.2 for all). It might be expected that if polymorphisms of GPIIIa do play a role in the risk of thrombosis these differences would affect receptor functions, such as ligand affinity or intracellular signalling, rather than the constituent level of expression of the protein.

The precise mechanism by which a single amino acid substitution at position 33 of GPIIIa might affect platelet fibrinogen binding (and hence aggregation) is unclear. This region of GPIIIa is not directly involved in binding either the ArgGlyAsp sequence on fibrinogen α-chains or the gamma-chain dodecapeptide[21]. However, some human antibodies specific for GPIIIa-Leu33 inhibit fibrinogen binding, possibly by preventing the necessary conformational change in GPIIb-IIIa required for the binding of fibrinogen[22]. The fact that substitution of proline for leucine results in the formation of an alloantigen indicates that a significant conformational change occurs which is being recognized by the immune system as non-self. The question whether this change in conformation is reflected in an altered function of in the GPIIb-GPIIIa heterodimer either by altering the affinity of the receptor for fibrinogen, or by affecting the mechanism of inside-out or outside-in signalling[23] can only be answered by further structure/function studies. The apparent selectivity of the effect, showing differences in the response to ADP but not to thrombin, would support the argument for an effect on receptor-mediated signalling pathways.

The whole blood flow cytometric assay used here does not involve separation or fixation steps and therefore avoids loss of platelets and fixation artefacts[17] and, because the platelets are not fixed, their response to agonist stimulation can also be measured in the presence of autologous plasma. The differences seen are unlikely to be caused by drug treatment as the patients were standardized to aspirin and a beta-blocker prior to study. Aspirin does not affect fibrinogen binding in this assay[24] and although beta-blockers cause a small increase in fibrinogen binding in vivo[18] this effect would be seen in all patients. Whilst abnormally high or low levels of plasma fibrinogen or platelet counts could alter fibrinogen binding in this assay[17], the patients in the present study all had levels of these parameters within the normal range and no correlation was found with the degree of fibrinogen binding.

Since the publication of the first case control study of 139 subjects[15] there has been considerable interest in the possibility that the GPIIIa-Pro33 may be a risk factor for thrombosis. However, most subsequent studies have failed to confirm the initially reported, strong association between GPIIIa-Pro33 and thrombotic risk. Three large case control studies, one on 451 U.K. subjects[25], one on a cohort of 704 individuals taken from the Physicians Health Study[26] and the third on 629 patients from the French/Irish ECTIM study[27], found no increased risk of myocardial infarction[25–27], stroke, or venous thrombosis[26]. Two further case control studies conducted in the U.K. and U.S.A.[29] of 133 and 324 subjects, respectively, also failed to find any association. However, in a U.K.-based study of 24 patients with myocardial infarction <47 years there was a significant association with GPIIIa-Pro33[30] and a weaker association in a group of 101 patients with an myocardial infarction below the age of 60[31], and a recent study of 318 patients undergoing coronary
revascularization found an increase in intracoronary stent thrombosis associated with the GPIIIa-Pro33 genotype\[32\]. Taken together with the current finding, this suggests that the GPIIIa-Pro33 +ve genotype may increase thrombotic risk when combined with other factors, such as early atherosclerotic disease\[15,30,31\] or mechanically-induced vascular injury\[32\]. It cannot be ruled out that in many of these studies the Pro33 variant of GPIIIa may have an adverse effect on survival, resulting in a bias toward the Leu-33 genotype in the sample study populations.

The present findings are supported by a recent report\[32\] of a study of 1336 subjects from the Framingham Offspring Study in which, despite the previous failure to detect an association between the polymorphic variants and thrombotic risk\[26\], significantly enhanced platelet aggregation was seen in response to ADP and to another ‘weak’ platelet agonist, epinephrine.

The fact that in this study differences were seen in the response of the platelets to ADP, but not thrombin, suggests that the increased risk of thrombosis may be associated with situations where ADP-mediated platelet activation is the primary factor; for example in shear-stress-induced activation; conditions that would be expected to be found in close proximity to an atherosclerotic plaque, or following stenting. Whilst the mechanisms for these differences require further investigation, these findings could argue for a selective use of inhibitors of ADP-mediated pathways in GPIIIa-Pro33+ve patients and may help to explain the beneficial effects of Ticlopidine in intracoronary stenting\[48\].

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References


