Platelet GP IIIa PlA Polymorphisms Display Different Sensitivities to Agonists

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Background—Both inherited predisposition and platelet hyperreactivity have been associated with ischemic coronary events, but mechanisms that support genetic differences among platelets from different subjects are generally lacking. Associations between the platelet PlA2 polymorphism of GP IIIa and coronary syndromes raise the question as to whether this inherited variation may contribute to platelet hyperreactivity.

Methods and Results—In this study, we characterized functional parameters in platelets from healthy donors with the PlA (HPA-1) polymorphism, a Leu (PlA1) to Pro (PlA2) substitution at position 33 of the GP IIIa subunit of the platelet GP IIb/IIIa receptor (integrin $\alpha_{IIb}\beta_3$). We studied 56 normal donors (20 PlA1,A1, 20 PlA1,A2, and 16 PlA2,A2). Compared with PlA1,A1 platelets, PlA2-positive platelets showed a gene dosage effect for significantly greater surface-expressed P-selectin, GP IIb/IIIa–bound fibrinogen, and activated GP IIb/IIIa in response to low-dose ADP. Surface expression of GP IIb/IIIa was similar in resting platelets of all 3 genotypes but was significantly greater on PlA2,A2 platelets after ADP stimulation ($P<0.003$ versus PlA1,A1; $P=0.03$ versus PlA1,A2). PlA1,A2 platelets were more sensitive to inhibition of aggregation by pharmacologically relevant concentrations of aspirin and abciximab.

Conclusions—PlA2-positive platelets displayed a lower threshold for activation, and platelets heterozygous for PlA alleles showed increased sensitivity to 2 antiplatelet drugs. These in vitro platelet studies may have relevance for in vivo thrombotic conditions. (Circulation. 2000;101:1013-1018.)

Key Words: platelets coronary disease polymorphisms inhibitors

Most acute ischemic coronary syndromes result from the formation of a platelet-rich thrombus at the site of a ruptured atherosclerotic plaque. Platelet hyperreactivity has been correlated with coronary events and mortality in patients with established coronary artery disease. Platelet aggregation is mediated through the binding of fibrinogen or von Willebrand factor (vWF) to the activated form of the platelet glycoprotein (GP) IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) receptor. Platelet membrane adhesive receptors are polymorphic, and recent reports of associations between the platelet PlA polymorphism of GP IIIa and ischemic coronary syndromes raise the question as to whether this genetic variation may contribute to platelet hyperreactivity. This is particularly important considering that $\approx 25\%$ of individuals of Northern European ancestry are PlA2-positive, with only $2\%$ being homozygous PlA2.

Standard light transmission (“turbidimetric”) platelet aggregometry is designed to detect platelet hypofunction in the evaluation of hemorrhagic conditions. This technique detects only aggregates of several hundred platelets and thus is insensitive to the early stages of platelet aggregation and may be suboptimal in discriminating platelet hyperreactivity. An additional concern regarding the use of platelet-rich plasma (PRP) in turbidimetric aggregometry is the loss of larger, high-density platelets during the centrifugation procedure. Whole-blood platelet aggregation measures electrical impedance caused by platelet thrombus formation on electrodes. However, this technique may suffer from nonreproducibility because of other causes of increased impedance, such as fibrin buildup on the electrodes. We have previously shown that a whole-blood flow cytometric analysis using epitope-dependent monoclonal antibodies is an extremely reliable measure of the platelet activation state. Using this assay and several others, we investigated whether the PlA2 polymorphism might contribute to the heterogeneity observed in platelet GP IIb/IIIa function by testing platelets from 56
normal donors, 16 of whom were Pl A2,A2. We found that Pl A2-positive platelets were hyperreactive and demonstrated an altered sensitivity to antiplatelet agents.

**Methods**

**Blood Collection**

After the Pl A genotype had been determined, repeat phlebotomy on healthy individuals was performed for functional studies when the donor was in a fasting state and had not taken medications for at least 10 days. The first 2 mL was discarded, then 100 mL of blood was drawn through a 19-gauge needle into 3.2% sodium citrate. Samples were included for functional studies only if there was maximal aggregation to arachidonic acid. In a few cases, not all assays could be performed on all 56 donors, usually because of insufficient blood sample.

**Monoclonal Antibodies**

Monoclonal antibody F26 is directed against a conformational change in fibrinogen bound to the GP IIb/IIIa complex.8 Monoclonal antibody LIBS1 is directed against a conformational change in the GP IIb/IIIa complex induced by fibrinogen binding.9 F26 and LIBS1 were FITC-conjugated as described.7 Peridinin chlorophyll protein (PerCP)–conjugated CD42a (Becton Dickinson) is a GP IX–specific monoclonal antibody. Phycoerythrin-conjugated monoclonal antibody CD62P (Becton Dickinson) is directed against P-selectin.10

**Whole-Blood Flow Cytometry**

Two- and 3-color whole-blood flow cytometry was performed by a modification of previously described methods,7 with the operator blinded to the genotype of the donor. Briefly, samples were incubated with CD42a-PerCP 2.5 μg/mL, followed by saturating concentrations of antibodies, followed by buffer or agonists. Samples were fixed in 1% formaldehyde, diluted in buffer, and sent at 4°C to the University of Massachusetts Medical Center. All samples were analyzed within 24 hours in an XL flow cytometer (Coulter) as described.7 In the 2-color assay, color compensation was not required because PerCP does not interfere with FITC. In the 3-color assay, stimulated or unstimulated normal donor samples labeled with test or isotype-matched control antibodies were prepared to establish appropriate color compensation.

**Quantification of Platelet Fibrinogen Binding, GP IIb/IIIa, and Platelet Fibrinogen and vWF**

PRP was prepared by centrifugation at 120g for 20 minutes. Functional studies were completed within 2.5 hours. We have previously described the FITC-labeled fibrinogen preparation, binding to platelets, and the method of converting fluorescence intensity data to the number of binding sites per cell.11 The bivalent form (IgG) of monoclonal antibody 7E3, directed against GP IIIa, was used in a radiometric assay at a concentration of 18 μg/mL to quantify the number of receptors per platelet as previously described.12 Fibrinogen and vWF were quantified from washed platelets by an ELISA technique from Accurate Chemical and Scientific Corp and American Diagnostica, Inc.

**Platelet Aggregation**

PRP was adjusted to 250 000 platelets/μL with autologous platelet-poor plasma for use in a BIO-DATA 4-channel platelet aggregometer. One hundred percent aggregation was the optical density obtained with platelet-poor plasma. The sample (450 μL) was preincubated at 37°C for 10 minutes. For the inhibition studies, aspirin was added for an additional 10 minutes. Because of variable response to epinephrine, the data for this agonist were used only if >60% aggregation was obtained with 10 μmol/L epinephrine.

![Figure 1](http://circ.ahajournals.org/)

**Table 1. Donor Characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>plA1,A1</td>
</tr>
<tr>
<td>No.</td>
<td>20</td>
</tr>
<tr>
<td>Male/female, n</td>
<td>10/10</td>
</tr>
<tr>
<td>Mean age, y±SEM</td>
<td>32±1.8</td>
</tr>
<tr>
<td>Current tobacco use, n</td>
<td>1</td>
</tr>
<tr>
<td>Mean PRP platelet count, cells/mL±SEM</td>
<td>±13 300</td>
</tr>
</tbody>
</table>

**IC₅₀ Determinations**

The 50% inhibitory concentration (IC₅₀) of aspirin was modeled for each subject individually. A sigmoid Emax model achieved the best fit for nearly all subjects according to an accepted model diagnostic criterion.13

**Statistics**

Data were analyzed by 1-way ANOVA and repeated-measures ANOVA. A Bonferroni-Dunn test was used for post hoc comparisons of individual means. A probability of <0.05 was considered significant. The IC₅₀ model parameters were not normally distributed. Accordingly, nonparametric methods were used for comparisons among (Kruskall-Wallis) and between (Mann-Whitney U test) genotype groups to determine statistical significance (2-sided P<0.05).

**Results**

**Donor Characteristics**

The characteristics of the blood donors are shown in Table 1 according to Pl A genotype. The groups were similarly matched, except that the Pl A2 homozygote group subjects were older than the other groups. There was no difference in platelet counts among the groups.

**Subthreshold Platelet Stimulation Studies**

Resting platelets that were Pl A2-positive bound significantly higher levels of CD62P (specific for P-selectin) than did Pl A-negative platelets (Pl A1,A1 versus Pl A1,A2, P=0.01; Pl A1,A1 versus Pl A2,A2, P=0.001), reflecting α-granule secretion (Figure 1). The F26 antibody showed greater binding to the Pl A2,A2
platelets than to the other genotypes. LIBS1 binding showed a trend toward greater binding to Pl A2-positive platelets (not shown), and a larger sample size may be necessary to detect a significant difference in LIBS1 binding to resting platelets. In addition, in resting platelets, perhaps the PlA2 conformation favors fibrinogen and F26 binding without LIBS1 exposure.

When blood was stimulated with low concentrations of ADP, a consistent PlA2-dependent increase in binding to CD62P, F26, and LIBS1 was observed (Figure 2). Compared with PlA1,A1 platelets, the homozygous PlA2,A2 platelets bound significantly more of all 3 antibodies on stimulation with both 0.5 µmol/L and 1.0 µmol/L ADP. At these same ADP concentrations, significant differences between PlA1,A1 and PlA2,A2 platelets were observed for the antibodies detecting GP IIb/IIIa activation: F26 and LIBS1. Similar variations were observed at 0.1 µmol/L ADP, although only F26 binding showed significant differences. These differences in binding of activation-dependent antibodies among the 3 genotypes were not due to differences in platelet size as assessed by forward light scatter in the flow cytometer (not shown).

We considered an effect of age on these findings, but the older 8 donors (mean age, 53.6 years) in the Pl A2,A2 group showed no significant differences from the younger 8 donors (mean age, 35.4 years) for any of the antibodies (Table 2). In addition, for every experiment in which there was a significant difference between the older half of the PlA2,A2 group and either Pl A1-positive group, there was also a significant difference between the younger half of the Pl A2,A2 group and either Pl A1-positive group (data not shown).

From these studies, we conclude that 2 copies of the PlA2 allele correlated with increased platelet reactivity, as determined by α-granule secretion (indicated by surface P-selectin) and the GP IIb/IIIa activation state (indicated by F26 and LIBS1 binding). A single copy of the PlA2 allele showed a consistent, but not statistically significant, association with increased platelet reactivity.

GP IIb/IIIa Quantification

We used several approaches to assess whether the increased binding of activation-dependent antibodies by PlA2 genotype was due to a higher absolute number of GP IIb/IIIa receptors. 7E3 is a GP IIb/IIIa–specific monoclonal antibody that can be used to quantify the number of receptors. No difference in the receptor density was observed by Pl A status in nonstimulated platelets (Figure 3). When stimulated with 20 µmol/L ADP, more GP IIb/IIIa receptors were detected on the Pl A2-positive platelets than on the Pl A2-negative platelets. Under the static conditions of these experiments, ADP would not have been expected to cause granule release, consistent with the lack of change observed in the PlA1,A1 platelets. These results suggested a lower threshold for degranulation in the
PI\textsuperscript{A2-positive platelets, consistent with the data in Figure 1, where a higher P-selectin expression was observed in PI\textsuperscript{A2-positive platelets. Age did not affect the increased 7E3 binding to PI\textsuperscript{A2,A2 platelets (Table 2). To confirm that \(\alpha\)-granule release was possible under the conditions of these experiments, we maximally stimulated platelets from 3 PI\textsuperscript{A1,A1} and 4 PI\textsuperscript{A1,A2} donors with 20 \(\mu\)mol/L thrombin receptor–activating peptide (TRAP) and observed the expected \(\approx30\%\) increase\textsuperscript{12} in GP IIb/IIIa receptor surface expression (maximal receptor numbers were 57 377 \pm 5486 for PI\textsuperscript{A1A1} and 57 510 \pm 6582 for PI\textsuperscript{A1,A2}) compared with unstimulated platelets. We also assessed binding of a GP IIb–specific monoclonal antibody (SZ22) and observed an equivalent binding to platelets of all 3 PI\textsuperscript{A} genotypes under both resting conditions and stimulation with 20 \(\mu\)mol/L TRAP (data not shown). Similar results with TRAP using 2 different antibodies make it unlikely that the difference in 7E3 binding to platelets of all 3 PI\textsuperscript{A} genotypes is due to a change in the affinity of 7E3 for the PI\textsuperscript{A} polymorphism. This conclusion is supported further by our findings in stable cell lines overexpressing the PI\textsuperscript{A1} or PI\textsuperscript{A2} forms of GP IIb/IIIa, which show equivalent binding of 7E3 Fab at concentrations >1 \(\mu\)g/mL (data not shown). We conclude that there is no difference in the total number of surface-accessible GP IIb/IIIa receptors among the 3 PI\textsuperscript{A} genotypes and that PI\textsuperscript{A1}–positive platelets possess a lower threshold for \(\alpha\)-granule release, with a corresponding increase in GP IIb/IIIa receptor density.

Maximal-Platelet-Stimulation Studies

The binding of exogenously added fibrinogen to maximally stimulated platelets was determined by use of 20 \(\mu\)mol/L ADP. Under these conditions, no significant differences were observed among the 3 genotypes (Table 3). Similarly, maximal stimulation with 20 \(\mu\)mol/L ADP and 20 \(\mu\)mol/L TRAP in platelet aggregometry studies negated any subthreshold difference (Table 3). Indeed, the only difference observed in aggregation studies was with 0.4 \(\mu\)mol/L epinephrine, in which PI\textsuperscript{A2,A2} platelets showed greater aggregation than PI\textsuperscript{A1-positive platelets. Finally, there were no significant differences among the PI\textsuperscript{A1A1} and PI\textsuperscript{A1,A2} genotypes in intracellular fibrinogen or vWF (Table 3).

Influence of PI\textsuperscript{A} Status on Aspirin and Abciximab Inhibition of ADP-Induced Platelet Aggregation

Aspirin is a mainstay in the treatment of coronary artery disease, and it inhibits epinephrine-induced platelet aggregation. We observed significant differences in platelet inhibition among PI\textsuperscript{A} genotypes at 2.5 and 5 \(\mu\)mol/L aspirin (Figure 4A), concentrations that are typically obtained in vivo.\textsuperscript{15} As we have previously reported,\textsuperscript{16} the PI\textsuperscript{A1,A2} platelets were the most sensitive to aspirin. Interestingly, PI\textsuperscript{A2,A2} platelets, which were not studied previously, were less sensitive than PI\textsuperscript{A1,A2} platelets and only slightly less sensitive than PI\textsuperscript{A1,A1} platelets. Abciximab is a monoclonal chimeric Fab fragment of 7E3 that blocks ligand binding to GP IIb/IIIa and has been beneficial in a number of coronary ischemic syndromes.\textsuperscript{17} As with aspirin, several concentrations of abciximab, within the range of concentrations that partially or completely block platelet aggregation,\textsuperscript{18} caused significant differences among genotypes in inhibiting aggregation (Figure 4B). There were statistically significant differences in the aspirin IC\textsubscript{50} (Table 4) among genotypes (\(P=0.024\)), with PI\textsuperscript{A2,A2} having the highest value relative to the other genotypes. Individual

### Table 2. Effect of Age on ADP-Induced Platelet Activation Within the PI\textsuperscript{A2,A2} Donors

<table>
<thead>
<tr>
<th>PI\textsuperscript{A2}</th>
<th>Age, y</th>
<th>Sex, F/M</th>
<th>1 (\mu)mol/L</th>
<th>20 (\mu)mol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Younger (n=8)</td>
<td>35.4±3.0</td>
<td>6/2</td>
<td>32.9±2.6</td>
<td>60.8±6.1</td>
</tr>
<tr>
<td>Older (n=8)</td>
<td>53.6±9.6</td>
<td>3/5</td>
<td>36.4±2.6</td>
<td>64.4±6.3</td>
</tr>
<tr>
<td>Pt</td>
<td>0.0002</td>
<td></td>
<td>0.49</td>
<td>0.63</td>
</tr>
</tbody>
</table>

*Values for different age groups are mean fluorescence units (as in Figure 2).
†Differences in mean age by the unpaired \(t\) test; other comparisons by repeated-measures ANOVA.

### Table 3. Platelet Functional Characteristics by PI\textsuperscript{A} Genotype

<table>
<thead>
<tr>
<th>PI\textsuperscript{A1}</th>
<th>PI\textsuperscript{A2}</th>
<th>PI\textsuperscript{A3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exogenous fibrinogen bound*†</td>
<td>18 795±1186 (n=20)</td>
<td>17 080±2118 (n=20)</td>
</tr>
<tr>
<td>Aggregation to 0.4 (\mu)mol/L epinephrine</td>
<td>22.6±6.5% (n=20)</td>
<td>16.4±5.4% (n=20)</td>
</tr>
<tr>
<td>Aggregation to 20 (\mu)mol/L ADP</td>
<td>81.4±1.0% (n=20)</td>
<td>81.0±1.8% (n=20)</td>
</tr>
<tr>
<td>Aggregation to 20 (\mu)mol/L TRAP</td>
<td>84.6±1.0% (n=20)</td>
<td>82.2±1.6% (n=20)</td>
</tr>
<tr>
<td>Total fibrinogen, mg/10\textsuperscript{11} platelets</td>
<td>11.4±2.33 (n=13)</td>
<td>12.4±2.27 (n=10)</td>
</tr>
<tr>
<td>Total platelet vWF, mU/mL</td>
<td>1.22±0.8±1.08×10\textsuperscript{10} (n=12)</td>
<td>1.73±0.8±1.57×10\textsuperscript{10} (n=10)</td>
</tr>
</tbody>
</table>

*No significant differences were present between any pairs of variables in any row, except aggregation to 0.4 \(\mu\)mol/L epinephrine, where \(P=0.03\) for PI\textsuperscript{A2} vs PI\textsuperscript{A1} and \(P=0.02\) for PI\textsuperscript{A3} vs PI\textsuperscript{A1}.
†Mean number of molecules bound by 20 \(\mu\)mol/L ADP and 100 \(\mu\)g/mL FITC-fibrinogen.
‡Platelets prepared without residual plasma also showed no difference in fibrinogen binding by PI\textsuperscript{A} genotype.
§Platelets could not be resuspended.
our studies demonstrate that PlA2-positive platelets, compared with PlA1,1A1 platelets, have a lower threshold for platelet activation, α-granule release, GP Ib/IIia activation, and fibrinogen binding. This "hyperreactive" state suggests that in vivo, PlA2-positive platelets may exert a greater thrombotic tendency than PlA1,1A1 platelets. We also observed a greater sensitivity to therapeutic concentrations of aspirin and abciximab in PlA1,2A2 platelets. This differential sensitivity to antiplatelet agents may have potential clinical implications whereby specific antiplatelet therapy may best be tailored according to a patient’s PlA genotype. On the basis of the findings in this mechanistic study, further studies are warranted to examine the in vivo effects of antiplatelet agents according to inherited variations in platelet adhesive receptors.

A panel of monoclonal antibodies reporting the activation state of platelets and GP Ib/IIia consistently demonstrated a higher degree of activation in PlA2-positive platelets. PlA2-positive platelets had a lower threshold for α-granule release, as shown by increased CD62P binding (Figures 1 and 2) and 7E3 binding observed with 20 μmol/L ADP (Figure 3). Presumably, the increased fibrinogen occupancy of PlA2-positive GP Ib/IIia receptors in the resting state or on weak agonist stimulation results in greater outside-in signaling and subsequent degranulation and P-selectin expression. The F26 antibody displayed an increased binding to ADP-stimulated PlA2-positive platelets and showed greater sensitivity than LIBS1 at detecting these activation differences. We observed both an increased LIBS1 binding in resting PlA2-positive platelets and a consistently greater activation of PlA1,2A1 platelets compared with PlA1,1A1 platelets, but statistical significance was not reached in either case. A larger sample size may be necessary to detect true differences. Our data were consistent with an age independence of the PlA2 effect, but because of the relatively small numbers of subjects compared when the PlA2,A2 group was divided in half (Table 2), further investigation in a larger population is warranted. Nevertheless, our findings are strongly supported by Feng et al.,19 who showed that age did not affect PlA2-induced platelet hyperreactivity among 1422 subjects.

Light transmission platelet aggregometry continues to be used as the "gold standard" of platelet function assays. However, the typical concentrations of agonists used in platelet aggregation studies may not be optimal for detecting platelet hyperreactivity. The only significant difference in aggregation by PlA status in our study was with low-dose epinephrine as an agonist, a finding consistent with the low-dose ADP data in our whole-blood assay (Figure 2) and observations that less epinephrine is required for aggregating PlA2-positive than PlA2-negative platelets.19 Platelets display an "all-or-none" activation response,20 which may explain why strong agonists were able to bypass or overcome the PlA2-dependent difference seen with weak agonists (Table 3).

In addition, because thrombogenesis under high shear rates typically proceeds via GP Ib/IIia–ligand interactions after platelets have adhered to the subendothelium via vWF,21 differences in the activation state of GP Ib/IIia or the threshold for granule release may not be apparent in the aggregometer, where low shear forces and repetitive interactions may provide sufficient time for bonds to form.

**Table 4. Effect of Inhibitors by Genotype**

<table>
<thead>
<tr>
<th></th>
<th>PlA1,1A1</th>
<th>PlA1,2A2</th>
<th>PlA2,2A2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IC50 for aspirin, μmol/L</strong></td>
<td>13.1±3.7 (n=17, 7M, 10F)</td>
<td>7.4±2.5 (n=13, 4M, 9F)</td>
<td>14.0±2.1 (n=15, 6M, 9F)</td>
<td>0.06 0.14 0.015</td>
</tr>
<tr>
<td><strong>IC50 for abciximab, μg/mL</strong></td>
<td>2.27±0.19 (n=20, 10M, 10F)</td>
<td>1.90±0.21 (n=20, 10M, 10F)</td>
<td>2.13±0.14 (n=14, 6M, 8F)</td>
<td>0.09 0.33 0.046</td>
</tr>
</tbody>
</table>
Interestingly, heterozygous platelets showed a greater sensitivity to 2 platelet inhibitors: aspirin and abciximab. It is not clear why the PlA2,A2 subjects responded to the inhibitors in a manner more like the PlA1,A1 group. Receptor clustering augments GP IIb/IIIa–mediated signaling, and perhaps such clustering may be inhibited in heterozygous platelets such that they are more susceptible to inhibition by aspirin or abciximab. For example, in studies with GP IIb/IIIa–expressing cell lines, we have found increased adhesion in PlA2-expressing cells compared with PlA1-expressing cells that is mediated through differences in outside-in signaling, and perhaps the combination of both allele products dominantly inhibits this component of receptor-mediated cell activation. The concentrations of both aspirin and abciximab used in these experiments were within the therapeutic range achieved in vivo. Thus, the significant differences in inhibition observed at 2.5 and 5.0 μmol/L aspirin and 1.25 and 1.5 μg/mL abciximab may affect either beneficial (antithrombotic) or adverse (hemorrhagic) in vivo effects. It will be interesting to see whether other structurally different platelet or GP IIb/IIIa antagonists display variable inhibitory activity by PlA genotype. Nevertheless, our data should be interpreted cautiously until they are confirmed in a larger series. In the meantime, future clinical epidemiology studies of platelet genetic variations and cardiovascular disease would be wise to consider possible treatment effects.

It is difficult to extrapolate these PlA-dependent differences in platelet function to the clinical condition, because platelet behavior in young, healthy donors is likely to be quite different from that in older patients with coronary artery disease, in which numerous other effects (cardiovascular medications, hormonal alterations, serum lipids, tobacco use, injured vessel wall, etc) affect platelet physiology. But because GP IIb/IIIa is the most abundant receptor on human blood platelets (≈80 000 copies per platelet) and plays a central role in formation of a platelet thrombus, even a subtle functional alteration could have profound effects over the lifetime of a patient.

Acknowledgments

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References


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