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.1 Platelet hyperreactivity has been correlated with coronary events and mortality in patients with established coronary artery disease.2 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 PlA2 polymorphism of GP IIIa and ischemic coronary syndromes3,4 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 PlA2.5

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.6 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.7 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 PlA2,A2. We found that PlA2-positive platelets were hyperreactive and demonstrated an altered sensitivity to antiplatelet agents.

**Methods**

**Blood Collection**

After the PlA 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. Monoclonal antibody LIBS1 is directed against a conformational change in the GP IIb/IIIa complex induced by fibrinogen binding. F26 and LIBS1 were FITC-conjugated as described. 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.

**Whole-Blood Flow Cytometry**

Two- and 3-color whole-blood flow cytometry was performed by a modification of previously described methods, 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 by overnight delivery to the Center for Platelet Function Studies at the University of Massachusetts Medical Center. All samples were analyzed within 24 hours in an XL flow cytometer (Coulter) as analyzed within 24 hours in an XL flow cytometer (Coulter). The sample was bivocal, and 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. 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. 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.

**Subthreshold Platelet Stimulation Studies**

Resting platelets that were PlA2-positive bound significantly higher levels of CD62P (specific for P-selectin) than did PlA2-negative platelets (PlA1,A1 versus PlA1,A2, P = 0.01; PlA1,A1 versus PlA2,A2, P = 0.001), reflecting α-granule secretion (Figure 1). The F26 antibody showed greater binding to the PlA2,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 of 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 PlA2,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 PlA1-positive group, there was also a significant difference between the younger half of the PlA2,A2 group and either PlA1-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 allele 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 status in nonstimulated platelets (Figure 3). When stimulated with 20 μmol/L ADP, more GP IIb/IIIa receptors were detected on the PlA2 positive platelets than on the PlA2-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
PLA2-positive platelets, consistent with the data in Figure 1, where a higher P-selectin expression was observed in PL(A2) positive platelets. Age did not affect the increased 7E3 binding to PL(A2) platelets (Table 2). To confirm that α-granule release was possible under the conditions of these experiments, we maximally stimulated platelets from 3 PlA1,A1 and 4 PlA1,A2 donors with 20 μmol/L thrombin receptor–activating peptide (TRAP) and observed the expected ≈30% increase in GP IIb/IIIa receptor surface expression (maximal receptor numbers were 57 377±5 486 for PlA1,A1 and 57 510±6 6582 for PlA1,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 PLA genotypes under both resting conditions and stimulation with 20 μmol/L TRAP (data not shown).

Similar results with TRAP using 2 different antibodies made it unlikely that the difference in 7E3 binding to platelets of all 3 PLA genotypes under both resting conditions was simply due to an altered affinity of 7E3 for the PLA2 polymorphism. This conclusion is supported further by our findings in stable cell lines overexpressing the PLA1 or PLA2 forms of GP IIb/IIIa, which show equivalent binding of 7E3 Fab at concentrations >1 μg/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 PLA genotypes and that PLA2-positive platelets possess a lower threshold for α-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 μmol/L ADP. Under these conditions, no significant differences were observed among the 3 genotypes (Table 3). Similarly, maximal stimulation with 20 μmol/L ADP and 20 μmol/L TRAP in platelet aggregometry studies negated any subthreshold difference (Table 3). Indeed, the only difference observed in aggregation studies was with 0.4 μmol/L epinephrine, in which PLA2,A2 platelets showed greater aggregation than PLA1,A1, positive platelets. Finally, there were no significant differences among the PLA1,A1 and PLA1,A2 genotypes in intracellular fibrinogen or vWF (Table 3).

Influence of PLA 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 PLA genotypes at 2.5 and 5 μmol/L aspirin (Figure 4A), concentrations that are typically obtained in vivo. As we have previously reported, the PLA1,A2 platelets were the most sensitive to aspirin. Interestingly, PLA2,A2 platelets, which were not studied previously, were less sensitive than PLA1,A2 platelets and only slightly less sensitive than PLA1,A1 platelets. Abciximab is a monovalent chimeric Fab fragment of 7E3 that blocks ligand binding to GP IIb/IIIa and has been beneficial in a number of coronary ischemic syndromes. As with aspirin, several concentrations of abciximab, within the range of concentrations that partially or completely block platelet aggregation, caused significant differences among genotypes in inhibiting aggregation (Figure 4B). There were statistically significant differences in the aspirin IC₅₀ (Table 4) among genotypes (P=0.024), with PLA2,A2 having the highest value relative to the other genotypes.

Table 2. Effect of Age on ADP-Induced Platelet Activation Within the PLA2,A2 Donors

<table>
<thead>
<tr>
<th>PLA2,A2</th>
<th>Age, y</th>
<th>Sex, F/M</th>
<th>CD62P*</th>
<th>F26*</th>
<th>LIBS1*</th>
<th>7E3 Binding Sites</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>
<td>58.0±4.0</td>
<td>53 290±20 57</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>
<td>63.1±4.8</td>
<td>53 870±28 59</td>
</tr>
<tr>
<td>P</td>
<td>0.0002</td>
<td>0.49</td>
<td>0.63</td>
<td>0.50</td>
<td>0.90</td>
<td></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 PLA Genotype

<table>
<thead>
<tr>
<th>PLA1,A1</th>
<th>PLA1,A2</th>
<th>PLA2,A2</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 μmol/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 μmol/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 μmol/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/10¹¹ platelets</td>
<td>11.4±2.33 (n=13)</td>
<td>12.4±2.27 (n=10)</td>
</tr>
<tr>
<td>Total platelet vWF, μU/mL</td>
<td>1.72×10⁻⁹±1.08×10⁻⁹ (n=12)</td>
<td>1.73×10⁻⁹±1.57×10⁻⁹ (n=10)</td>
</tr>
</tbody>
</table>

†Platelets prepared without residual plasma also showed no difference in fibrinogen binding by PLA genotype.
‡Platelets could not be resuspended.
A panel of monoclonal antibodies reporting the activation state of platelets and GP Ib/IIa 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/IIa 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,A2 platelets compared with PlA1,A1 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 platelets and a consistently greater activation of PlA1,A2 platelets compared with PlA2,A2 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/IIa–ligand interactions after platelets have adhered to the subendothelium via vWF, 21 differences in the activation state of GP Ib/IIa 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>p&lt;sub&gt;1,A1&lt;/sub&gt;</th>
<th>p&lt;sub&gt;1,A2&lt;/sub&gt;</th>
<th>p&lt;sub&gt;2,A2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt; as aspirin, μmol/L</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>
</tr>
<tr>
<td><strong>P</strong></td>
<td>0.06</td>
<td>0.14</td>
<td>0.015</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt; as abciximab, μg/mL</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>
</tr>
<tr>
<td><strong>P</strong></td>
<td>0.09</td>
<td>0.33</td>
<td>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 PI A2, expressing cells compared with PI A1-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 μmol/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 Pl A 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 Pl A-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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