Platelet GP IIIa PlA Polymorphisms Display Different Sensitivities to Agonists

Alan D. Michelson, MD; Mark I. Furman, MD; Pascal Goldschmidt-Clermont, MD; Mary Ann Mascelli, PhD; Craig Hendrix, MD; Lindsay Coleman, BS; Jeanette Hamlington, BS; Marc R. Barnard, MS; Thomas Kickler, MD; Douglas J. Christie, PhD; Sourav Kundu, PhD; Paul F. Bray, MD

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
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.8 Monoclonal antibody LIBS1 is directed against a conformational change in the fibrinogen bound to the GP IIb/IIIa complex. F26 and LIBS1 were FITC-conjugated as described.9 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 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 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.

Results

Donor Characteristics
The characteristics of the blood donors are shown in Table 1 according to PlA genotype. The groups were similarly matched, except that the PlA2 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 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.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>prA1,A1</th>
<th>prA1,A2</th>
<th>prA2,A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>20</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>Male/female, n</td>
<td>10/10</td>
<td>10/10</td>
<td>7/9</td>
</tr>
<tr>
<td>Mean age, y ± SEM</td>
<td>32 ± 1.8</td>
<td>30.5 ± 1.8</td>
<td>44.5 ± 2.9</td>
</tr>
<tr>
<td>Current tobacco use, n</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Mean PRP platelet count, cells/mL ± SEM</td>
<td>390 400</td>
<td>395 500</td>
<td>390 700</td>
</tr>
<tr>
<td>Mean CD62P (PerCP) ± SEM</td>
<td>±13 300</td>
<td>±24 700</td>
<td>±19 200</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).

Figure 1. Analysis of platelet activation state under resting conditions using whole-blood flow cytometry. CD62P detects P-selectin. F26 detects a conformation of fibrinogen that is bound to GP IIb/IIIa. Numbers of donors used were 19 PlA1,A1, 18 to 19 PlA1,A2, and 14 to 15 PlA2,A2. Error bars indicate SEM. Numbers above bars indicate probability value for differences between 2 means.
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,A2 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 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 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
PIA2-positive platelets, consistent with the data in Figure 1, where a higher P-selectin expression was observed in PI A2-positive platelets. Age did not affect the increased 7E3 binding to PI A2,A2 platelets (Table 2). To confirm that α-granule release was possible under the conditions of these experiments, we maximally stimulated platelets from 3 PI A1,A1 and 4 PI A1,A2 donors with 20 μmol/L thrombin receptor–activating peptide (TRAP) and observed the expected positive platelets. Age did not affect the increased 7E3 binding to PI A2,A2 platelets (Table 2). To confirm that positive platelets possess a lower threshold for activation peptide (TRAP) and observed the expected binding to PI A2,A2 platelets (Table 2). To confirm that positive platelets, consistent with the data in Figure 1, where a higher P-selectin expression was observed in PI A2-positive platelets. Age did not affect the increased 7E3 binding to PI A2,A2 platelets (Table 2). To confirm that α-granule release was possible under the conditions of these experiments, we maximally stimulated platelets from 3 PI A1,A1 and 4 PI A1,A2 donors with 20 μmol/L thrombin receptor–activating peptide (TRAP) and observed the expected positive platelets. Age did not affect the increased 7E3 binding to PI A2,A2 platelets (Table 2). To confirm that positive platelets possess a lower threshold for

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

<table>
<thead>
<tr>
<th>PI A2,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±2057</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±2859</td>
</tr>
<tr>
<td>Pt</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 PI A Genotype**

<table>
<thead>
<tr>
<th>PI A1</th>
<th>PI A2,A2</th>
<th>PI A2,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, mU/mL</td>
<td>1.22×10−9±1.08×10−9 (n=12)</td>
<td>1.73×10−9±1.57×10−9 (n=10)</td>
</tr>
</tbody>
</table>

*Mean number of molecules bound by 20 μmol/L ADP and 100 μg/mL FITC-fibrinogen.

†Platelets prepared without residual plasma also showed no difference in fibrinogen binding by PI A genotype.

‡Platelets could not be resuspended.

**Influence of PI 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 A genotypes at 2.5 and 5 μmol/L aspirin (Figure 4A), concentrations that are typically obtained in vivo. As we have previously reported,16 the PI A1,A2 platelets were the most sensitive to aspirin. Interestingly, PI A2,A2 platelets, which were not studied previously, were less sensitive than PI A1,A1 platelets and only slightly less sensitive than PI A1,A2 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.17 As with aspirin, several concentrations of abciximab, within the range of concentrations that partially or completely block platelet aggregation,18 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 PI A2,A2 having the highest value relative to the other genotypes. Individual
Our studies demonstrate that PI2-positive platelets, compared with PI1A1 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, PI2-positive platelets may exert a greater thrombotic tendency than PI1A1 platelets. We also observed a greater sensitivity to therapeutic concentrations of aspirin and abciximab in PI1A2 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 PI2 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 PI2-positive platelets. PI2-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 PI2-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 PI2-positive platelets and showed greater sensitivity than LIBS1 at detecting these activation differences. We observed both an increased LIBS1 binding in resting PI2-positive platelets and a consistently greater activation of PI1A2 platelets compared with PI1A1 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 PI2 effect, but because of the relatively small numbers of subjects compared when the PI2A2 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 PI2-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 PI2 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 PI2-positive than PI2-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 PI2-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.

**Figure 4.** Effect of aspirin and abciximab by PI2 genotype. Data are normalized to 100%, defined as aggregation achieved with no inhibitor. Inset, Probability values for concentrations of inhibi-

tors that showed significant differences; PI1A1 is abbreviated 1,1; PI1A2, 1,2; and PI2A2, 2,2. A, Aggregation performed with 2 μmol/L epinephrine. Numbers of donors used were 17 PI1A1, 13 PI1A2, and 14 PI2A2. Two PI1A1, 6 PI1A2, and 2 PI2A2 donors did not achieve ≥60% aggregation to epinephrine. B, Aggregation performed with 5 μmol/L ADP. Numbers of donors used were 20 PI1A1, 20 PI1A2, and 15 PI2A2.

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>IC50 for aspirin, μmol/L</th>
<th>IC50 for abciximab, μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI1A1</td>
<td>13.1±3.7 (n=17, 7M, 10F)</td>
<td>2.27±0.19 (n=20, 10M, 10F)</td>
</tr>
<tr>
<td>PI1A2</td>
<td>7.4±2.5 (n=13, 4M, 9F)</td>
<td>1.90±0.21 (n=20, 10M, 10F)</td>
</tr>
<tr>
<td>PI2A2</td>
<td>14.0±2.1 (n=15, 6M, 9F)</td>
<td>2.13±0.14 (n=14, 6M, 8F)</td>
</tr>
</tbody>
</table>

**P**

- PI1A1 vs PI1A2: 0.06
- PI1A1 vs PI2A2: 0.014
- PI1A2 vs PI2A2: 0.015
Interestingly, heterozygous platelets showed a greater sensitivity to 2 platelet inhibitors: aspirin and abciximab. It is not clear why the PlA2,2 group subjects responded to the inhibitors in a manner more like the PlA1,1 group. Receptor clustering augments GP IIb/IIIa-mediated signaling,22 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 PlA2,2 expressing cells compared with PlA1,1-expressing cells that is mediated through differences in outside-in signaling,23 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.15,18 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

This study was supported by grant HL-57488 from the National Institutes of Health and Frank Pearl. We are grateful to all volunteers who so generously gave of their time and blood and to Drs Mark H. Ginsberg and Harvey Gralnick for generously providing F26 and LIBS1.

References


Platelet GP IIIa PlA Polymorphisms Display Different Sensitivities to Agonists
Alan D. Michelson, Mark I. Furman, Pascal Goldschmidt-Clermont, Mary Ann Mascelli, Craig Hendrix, Lindsay Coleman, Jeanette Hamlington, Marc R. Barnard, Thomas Kickler, Douglas J. Christie, Sourav Kundu and Paul F. Bray

*Circulation*. 2000;101:1013-1018
doi: 10.1161/01.CIR.101.9.1013

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/101/9/1013

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation* is online at:
http://circ.ahajournals.org//subscriptions/