Increased Platelet Aggregability Associated With Platelet *GPIIIa* Pl^{A2} Polymorphism

The Framingham Offspring Study

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Abstract—The platelet glycoprotein IIb/IIIa (GP IIb/IIIa) plays a pivotal role in platelet aggregation. Recent data suggest that the Pl^{A2} polymorphism of GPIIIa may be associated with an increased risk for cardiovascular disease. However, it is unknown if there is any association between this polymorphism and platelet reactivity. We determined *GP IIIa* genotype and platelet reactivity phenotype data in 1422 subjects from the Framingham Offspring Study. Genotyping was performed using PCR-based restriction fragment length polymorphism analysis. Platelet aggregability was evaluated by the Born method. The threshold concentrations of epinephrine and ADP were determined. Allele frequencies of Pl^{A1} and Pl^{A2} were 0.84 and 0.16, respectively. The presence of 1 or 2 Pl^{A2} alleles was associated with increased platelet aggregability as indicated by incrementally lower threshold concentrations for epinephrine and ADP. For epinephrine, the mean concentrations were 0.9 μmol/L (0.9 to 1.0) for homozygous Pl^{A1}, 0.7 mmol/L (0.7 to 0.9) for the heterozygous Pl^{A1}/Pl^{A2}, and 0.6 μmol/L (0.4 to 1.0) for homozygous Pl^{A2} individuals, $P=0.009$. The increase in aggregability induced by epinephrine remained highly significant (P=0.007) after adjustment for covariates. For ADP-induced aggregation, the respective mean concentrations were 3.1 μmol/L (3.0 to 3.2), 3.0 μmol/L (2.9 to 3.2), and 2.8 μmol/L (2.4 to 3.3); $P=0.19$ after adjustment for covariates. Our findings indicate that molecular variants of the gene encoding *GP IIIa* play a role in platelet reactivity in vitro. Our observations are compatible with and provide an explanation for the reported association of the Pl^{A2} allotype with increased risk for cardiovascular disease. **(*Arterioscler Thromb Vasc Biol.* 1999;19:1142-1147.)**

Key Words: platelets ■ genetics ■ glycoprotein ■ epinephrine

Myocardial infarction results from the formation of a platelet-rich thrombus at the site of a ruptured coronary atherosclerotic plaque.[1,2] The platelet surface receptor glycoprotein *IIb/IIIa* (*GP IIb/IIIa*) plays a key role in the formation of such a thrombus by binding fibrinogen and von Willebrand factor. The importance of the *GP IIb/IIIa* receptor has been further supported by recent clinical trials in which *GP IIb/IIIa* antagonists have been shown to reduce restenosis rate after angioplasty[3] and also to reduce the morbidity and mortality associated with unstable angina,[4] high-risk coronary angioplasty,[5] and acute myocardial infarction.[6]

Although the Pl^{A1} and Pl^{A2} variants of *GP IIIa* have long been recognized as alloantigens and most frequently implicated in syndromes of immune-mediated platelet destruction, until recently little attention has been paid to their role in coronary heart disease. Weiss and colleagues[7] first reported that patients with acute coronary syndromes were more likely than were controls to carry the Pl^{A2} allele. The risk associated with Pl^{A2} was especially high for those aged 60 years or younger at the time of infarction. Recently, Walter and colleagues[8] reported that patients with the Pl^{A2} allele had an increased risk of coronary stent thrombosis compared with Pl^{A1} homozygous individuals. However, the association between the Pl^{A2} allele and cardiovascular disease has not been a consistent finding. Although Carter et al[9] supported the early findings of Weiss,[1] several other studies failed to detect the association,[10–15] including a large prospective study from the Physicians' Health Study.[10]

Importantly, the mechanism for the possibly increased risk has not been determined. We hypothesized that the Pl^{A2} allele might be associated with an increase in platelet aggregability and tested this hypothesis in the Framingham Offspring Study.

Received September 18, 1998; revision accepted November 3, 1998.

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***Arterioscler Thromb Vasc Biol.* is available at http://www.atvbaha.org**

Methods

Study Population

The study subjects were members of the Framingham Offspring Study, a long-term, prospective evaluation of risk factors for cardiovascular disease. The design and methodology of the Framingham Offspring Study have been described in detail elsewhere.[16] The participants were natural or adopted children of the original Framingham Heart Study subjects. For this study, we collected data from subjects that were consecutively examined between April 3, 1991 and June 29, 1995, during the fifth Offspring Study examination cycle.

Of the 3799 subjects who attended examination cycle 5, blood samples were collected from 3286 subjects for platelet aggregation analysis. For the present analysis, we excluded subjects who were not members of a sibship (n=1298) because linkage analysis was also performed. We also excluded subjects in whom platelet aggregation data would not be determinable because of treatment with anticoagulant or antiplatelet drugs (n=536). Finally, we excluded subjects in whom genotyping could not be successfully accomplished (n=30). A total of 1422 subjects fulfilled all inclusion criteria.

Determination of Platelet Aggregability

Blood samples were always obtained in the morning to avoid the circadian change of platelet aggregability. Blood was drawn in 3.8% sodium citrate solution (9:1). Platelet-rich plasma was separated by centrifugation for 10 minutes at 160*g*. Platelet aggregation was measured on a 4-channel aggregometer according to the method of Born.[17] The aggregation agents tested were epinephrine and ADP in varying concentrations (0.01 to 30 μmol/L), and a fixed concentration of arachidonic acid (1.6 μmol/L). The lowest concentrations of ADP and epinephrine required to produce a biphasic response with >50% aggregation (threshold concentration) were determined. A decreased threshold concentration indicates an increase in platelet aggregability. In addition, the presence or absence of an aggregation in response to arachidonic acid was determined.

Genotyping

To detect the substitution of cytosine for thymidine at position 1565 in exon 2 of the glycoprotein *IIIa* gene that is responsible for the Pl^{A2} polymorphism, we used a modified PCR-based restriction fragment length polymorphism (RFLP) analysis.[18] Genomic DNA was isolated from whole blood. Genomic DNA (10 to 20 ng in 5 mL volume) was incubated at 96°C for 3 minutes, followed by addition of master-mix (10 μL) to yield a final reagent concentration of 333 nmol/L for sense and antisense primer, 167 nmol/L of each of dATP, dTTP, dCTP, and dGTP, 2.5 mmol/L magnesium chloride, 50 mmol/L potassium chloride, 10 mmol/L Tris-HCl (pH 8.4 at 25°C), 0.1%Triton X-100, 0.02 mmol/L cresol red, and 83 mmol/L sucrose, as well as 0.15 U of *Taq* polymerase. The sequences of the sense and antisense primers were 5′tgggacttctctttgggctcctgacttac3′ and 5′ccttcagcagattctccttcaggtcac3′, respectively. DNA was amplified by 39 cycles of denaturing at 96°C for 20 seconds, annealing at 56°C for 40 seconds, and extension at 72°C for 30 seconds.

Restriction buffer (10 μL) was added to yield a final concentration of 10 mmol/L Tris-HCl, 5.5 mmol/L magnesium chloride, 12.5 mmol/L sodium chloride, 30 mmol/L potassium chloride, 0.4 mmol/L dithiothreitol, and 0.1% Triton X-100. The samples were incubated at 37°C with 4 U of restriction endonuclease *Msp*I overnight. This step was then repeated for complete digestion. In the presence of the Pl^{A2} allele, but not the Pl^{A1} allele, the 82 base pair (bp) amplification product was cleaved into fragments of 39 bp and 43 bp.

*Msp*I digested amplification product (8 μL) was loaded onto 2% agarose gel slabs containing 40 mmol/L Tris acetate and 2 mmol/L EDTA. Samples were size-fractionated at 6 V/cm for 30 minutes. Bands were visualized after staining with ethidium bromide by 300 nm UV transillumination. PCR results were scored without knowledge of platelet aggregability results. When there was any ambiguity, genotyping was repeated. Ninety-eight percent of the subjects were successfully genotyped.

Statistical Analysis

Demographic and clinical characteristics were compared among genotype groups by one-way ANOVA or by χ^2 test. The χ^2 test was also used to compare the observed allele and genotype frequencies against Hardy–Weinberg equilibrium prediction. Data on epinephrine and ADP threshold concentrations were log-transformed and compared among genotype groups by one-way ANOVA[19] as well as the nonparametric Kruskall–Wallis test. Post hoc pairwise comparisons among genotypes were performed using Scheffe's adjustment. Multiple regression was used to adjust for age, sex, body mass index (BMI), diabetes, triglyceride, total cholesterol and HDL cholesterol, the presence of cardiovascular disease (CVD), menopausal status, and estrogen replacement status.[19,20] Separate models for recessive, dominant, and additive genetic effects were evaluated with use of appropriate dummy variables. Generalized estimating equation algorithms were used to correct for intrafamily correlations.[21] Data on platelet aggregation were expressed as geometric mean±95% confidence interval. A value of $P<0.05$ was regarded as statistically significant.

Finally, a test of genetic linkage based on excess allele sharing for the quantitative traits (epinephrine and ADP threshold concentrations) with *GP IIIa* genotype was carried out, using SIBPAL version 2.7 of S.A.G.E. (1996).[22,23] This program provides an estimate of the proportion of alleles identically shared by descent at the *GP IIIa* locus using the sibpairs under study. Under this algorithm, linkage between marker and phenotype results in a negative value for the slope of the regression of the squared trait difference on the estimated proportion of alleles.

Results

Subject Characteristics (Table 1)

There were no significant differences among individuals within each genotype group for age, sex, BMI, diabetes mellitus, smoking, CVD, hypertension, triglyceride level, total and HDL cholesterol levels, or alcohol consumption. The allele frequencies of Pl^{A1} and Pl^{A2} were 0.84 and 0.16, respectively, and are in accord with those predicted by the Hardy–Weinberg equilibrium ($P=0.44$).

The genotype frequencies were similar between subjects excluded from the present analysis in whom genotyping was performed and those included in the present analysis. The frequencies of Pl^{A1} homozygous, heterozygous and Pl^{A2} homozygous were 72.9%, 24.3%, and 2.8% among subjects excluded, and 71.5%, 26.0%, and 2.5%, respectively, among subjects included in the present analysis ($P=0.74$).

Pl^A Polymorphism and Platelet Aggregability: Association Analysis (Table 2)

Epinephrine-Induced Platelet Aggregation

The presence of 1 or 2 Pl^{A2} alleles was associated with an incrementally lower threshold concentration for epinephrine-induced aggregation (unadjusted ANOVA $P=0.009$ and Kruskall–Wallis $P=0.0008$). This increase in platelet aggregability associated with the Pl^{A2} allele remained significant (ANOVA, $P=0.007$) after adjustment for age, sex, BMI, diabetes, triglyceride, total and HDL cholesterol, presence of CVD, menopausal status, and estrogen replacement therapy. There was no difference in results of analyses which included or excluded subjects with CVD.

Post hoc analysis (Scheffe's test) was performed to compare genotype group pairwisely. The difference in epinephrine threshold concentration between Pl^{A1} homozygous and Pl^{A1}/Pl^{A2} heterozygous subjects was significant, $P=0.02$. Because of a small sample size in the Pl^{A2} homozygous group (n=36), the difference between Pl^{A2} homozygous and Pl^{A1}

TABLE 1. Demographic Characteristics*

	Pl^{A1}/Pl^{A1}	Pl^{A1}/Pl^{A2}	Pl^{A2}/Pl^{A2}	*P*
Number	n=1017	n=369	n=36	—
Sex (% male)	46	46	47	0.98
Age	53.4±0.3	54.0±0.5	51.4±1.7	0.30
Hypertension (%)	33	29	33	0.38
Cardiovascular disease (%)	6.7	7.9	8.3	0.72
Diabetes (%)	6.2	5.2	11.1	0.34
Smoker (%)	21	16	11	0.08
BMI (kg/m^2)	27.4±0.2	27.9±0.3	26.8±0.9	0.24
Triglyceride (mmol/L)	1.66±0.03	1.57±0.06	1.54±0.19	0.40
Total cholesterol (mmol/L)	5.28±0.03	5.33±0.05	5.35±0.16	0.70
HDL cholesterol (mmol/L)	1.27±0.01	1.32±0.02	1.27±0.06	0.23
Alcohol (oz/wk)	2.9±0.1	2.6±0.2	1.4±0.7	0.06

*Data are expressed as mean±SEM or percentages.

homozygous or Pl^{A1}/Pl^{A2} heterozygous subjects were statistically insignificant ($P=0.18$ and 0.69, respectively).

Regression models with dummy variables were used to test different modes of genetic transmission, in each case accounting for the above-mentioned possible confounds. The additive model (ie, a gene-dose model) yielded the best fit with $P=0.002$, followed by the dominant model ($P=0.003$). But in the recessive model, no statistically significant effect was seen ($P=0.16$). The threshold concentration of epinephrine decreased by 19% per "dose" of Pl^{A2} allele (by 35% for Pl^{A2} homozygous) relative to the Pl^{A1} homozygote.

ADP-Induced Platelet Aggregation

There was a trend toward the Pl^{A2} allele being associated with a decreased threshold concentration for ADP, which was directionally consistent with the results seen with epinephrine-induced aggregation. However, the differences observed were not statistically significant (ANOVA, $P=0.48$; Kruskall–Wallis test, $P=0.23$); after adjustment for covariates, $P=0.19$ (ANOVA).

Pl^A Polymorphism and Platelet Aggregability: Linkage Analyses Result

A negative regression coefficient (−0.1926), consistent with genetic linkage but not statistically significant ($P=0.35$), was observed for epinephrine-induced platelet aggregation. The regression coefficient for ADP threshold concentration was 0.2777 ($P=0.60$). The heterozygosity index of this dimorphic marker was 0.27.

Contribution of Genetic and Traditional Risk Factors to Platelet Aggregation (Table 3)

In the model for epinephrine-induced aggregation, sex accounted for 2.7% of the variance ($P<0.0001$), triglyceride 1.1% ($P<0.0001$), *GP IIIa* genotype 0.7% ($P=0.007$), and age 0.5% ($P=0.08$). The remaining variables contributed <0.2% each.

In the model for ADP-induced aggregation, sex accounted for 3.1% of the variance ($P<0.0001$), age 0.9% ($P=0.003$), triglyceride 0.8% ($P=0.0006$), HDL-cholesterol 0.5% ($P=0.006$), hormone replacement therapy 0.3% ($P=0.03$), and *GP IIIa* genotype 0.2% ($P=0.21$). The remaining variables contributed <0.2% each.

Discussion

In the Framingham Offspring Study, the presence of 1 or 2 Pl^{A2} alleles of the platelet GP IIIa receptor was associated with an incrementally lower platelet threshold concentration in response to epinephrine and a trend toward lower threshold concentration in response to ADP. The increase in aggregability induced by epinephrine remained highly significant after adjustment for covariates. For epinephrine-induced aggregation, *GPIIIa* genotype explained 0.7% of the variance, while age, sex, and triglyceride accounted for an additional 4.3% of the variance.

GP IIIa Polymorphism and CVD

The familial clustering of coronary heart disease and the presence of a higher concordance in mortality among monozygotic twins compared with dizygotic twins suggest an important pathogenic role for genetic factors.[24] Although a small proportion of coronary heart disease can be attributed to single gene defects (eg, familial hypercholesterolemia or homocystinuria), the nature of additional contributing genetic factors remains largely unknown. Because platelets play a central role in the pathogenesis of acute CVD, it is possible that inherited platelet variants may contribute to CVD risk. Knowledge of such variants and their phenotypic expression may lead to progress in coronary disease risk assessment and therapeutic intervention.

TABLE 2. Platelet Aggregability Induced by Epinephrine and ADP

	Pl^{A1}/Pl^{A1}	Pl^{A1}/Pl^{A2}	Pl^{A2}/Pl^{A2}	*P**
Epinephrine (μmol/L)	0.9 (0.9–1.0)	0.7 (0.7–0.9)	0.6 (0.4–1.0)	0.007
ADP (μmol/L)	3.1 (3.0–3.2)	3.0 (2.9–3.2)	2.8 (2.4–3.3)	0.190

**P* values are ANOVA, adjusted for age, sex, BMI, diabetes, triglyceride, total and HDL cholesterol, cardiovascular disease, menopausal status, and estrogen replacement therapy.

TABLE 3. Contribution of Genetic and Traditional Risk Factors to Platelet Aggregation

	Factors	Contribution	*P* Value	Note
Epinephrine-induced aggregation*	Sex	2.7%	<0.0001	Female associated with an increased aggregability
	Triglyceride	1.1%	<0.0001	Lower triglyceride associated with an increased aggregability
	GPIIIa genotype	0.7%	0.007	*PlA*2 allele associated with an increased aggregability
ADP-induced aggregation†	Sex	3.1%	<0.0001	Female associated with an increased aggregability
	Age	0.9%	0.003	Increased age associated with an increased aggregability
	Triglyceride	0.8%	0.0006	Lower triglyceride associated with increased aggregability
	HDL-cholesterol	0.5%	0.006	Lower levels associated with increased aggregability
	HRT‡	0.3%	0.03	Therapy associated with increased aggregability
	GP IIIa genotype	0.2%	0.21	*PlA*2 allele associated with increased aggregability

*In the epinephrine-induced aggregation, the remaining variables, including age, CVD, BMI, diabetes, total and HDL cholesterol, menopausal status, and HRT were not significantly associated with platelet aggregability. †In the ADP-induced platelet aggregation, CVD, BMI, diabetes, total cholesterol levels, and menopausal status, were not significantly associated with platelet aggregability. ‡HRT indicates hormone replacement therapy.

Weiss and colleagues[7] showed that patients with acute coronary syndromes were more likely than were controls to carry the Pl^{A2} allele. In their study, the prevalence of the Pl^{A2} allele was 2.1 times higher in the patients than among the controls. These findings, coupled with an anecdotal report about the sudden death of a 28-year-old Olympic skater who had severe coronary artery disease and carried the Pl^{A2} allele, but no other traditional risk factors, resulted in the Pl^{A1}/Pl^{A2} dimorphism receiving widespread attention.[25] Further studies of this genetic marker are warranted because, although there has been some support for the findings of Weiss et al,[9,26] results from several other groups found no association between the Pl^{A2} allele and CVD, including an analysis by Ridker et al of the Physicians' Health Study.[10–15]

GP IIIa Polymorphism and Platelet Aggregability

Platelet *GP IIb/IIIa* is the most abundant platelet receptor, with an estimated 50 000 copies per cell.[27] It is present in the platelet membrane as a heterodimeric complex whose formation requires the presence of divalent cations. The receptor is highly polymorphic and has long been recognized as having alloantigens.[28] Pl^{A} alloantigens have been most frequently considered for their role in syndromes of immune-mediated platelet destruction, such as post-transfusion purpura and neonatal alloimmune thrombocytopenic purpura.[28] Newman and colleagues[18] identified the molecular basis of this polymorphism. The Pl^{A1}-allotype carries a leucine at position 33 of glycoprotein *IIIa* whereas the Pl^{A2}-allotype has a proline at position 33, because of a thymidine to cytosine substitution at 1565 in exon 2 of the glycoprotein *IIIa* gene.

The functional influence of the *GP IIIa* polymorphism on platelet reactivity is largely unknown. Using epinephrine as a platelet agonist, we found that the presence of the Pl^{A2} allele was associated with heightened platelet aggregability. Furthermore, the Pl^{A2}-associated increase in aggregability remained significant after adjustment for traditional risk factors that could influence platelet aggregability. The effect of the *GP IIIa* polymorphism on epinephrine-induced aggregation is in accordance with an additive model, with threshold concentration decreased by 19% per "dose" of Pl^{A2} allele (35% for Pl^{A2} homozygous) as compared with the Pl^{A1} homozygote. Using multiple regression analysis, we found that the polymorphism explained a small, but significant, percentage of variance of aggregability induced by epinephrine.

The platelet Pl^{A} antigen system is not in the 2 putative RGD sequence binding regions of *GP IIIa*, which are located within residues 107 to 179 and 211 to 222 from the amino terminal, respectively.[29,30] However, according to Calvete,[31] the Leu33/Pro33 polymorphism is enclosed within a small 13–amino acid loop formed by the pairing of Cys26 with Cys38. In addition, a long-range disulfide bond linking Cys5 and Cys435 has been identified which could bring the amino-terminal region of *IIIa*, including the small loop that contains the Pl^{A} polymorphic residue, into immediate proximity with the binding regions of *IIIa*.[29,30] Because of proline's unique structure, proline substitutions are well recognized for their propensity to induce conformational changes. Such changes can create alloantigenic determinants recognizable by T cells and B cells and induce the production of antibody.[32] The conformational changes could also influence activation of the *GP IIb/IIIa* receptor and alter platelet aggregability. Equally possible, the Pl^{A} polymorphism may be in linkage disequilibrium with other as yet undefined molecular variants of the gene that influence platelet reactivity.

Goldschmidt-Clermont and colleagues[33] quantitated fibrinogen binding to platelets of different allotypes. The investigators found that platelets with the Pl^{A2} allele bound significantly less fibrinogen than did platelets that were homozygous for Pl^{A1}. Differences in methodology used to

evaluate platelet reactivity in the study make it difficult to compare with our data. Additional larger and more comprehensive investigations will be required to resolve the issue. In a recent study, Cooke et al[34] found that platelets with the Pl^{A2} allele were more sensitive to aspirin inhibition.

Finally, we studied the relationship between the Pl^{A} polymorphism and an intermediate phenotype (ie, platelet aggregability), rather than coronary heart disease. Although it has not been demonstrated that epinephrine-induced platelet aggregation is an independent risk factor for coronary heart disease, there is considerable evidence linking platelet reactivity to CVD.[35–37] In the Framingham Heart Study, we will prospectively follow the population to determine whether epinephrine-induced platelet aggregability and the Pl^{A2} allele are risk factors for CVD.

Limitations of the study

First, our analysis was based on a single measurement of platelet aggregation. However, any random variation or misclassification would introduce bias that favors the null hypothesis and an underestimation of the genetic contribution to platelet aggregability. Additional measures of platelet function should be evaluated in future studies. Second, our analysis was based on the subset of Framingham subjects in whom both genotype and phenotype data were available. However, the genotype distribution was similar between subjects excluded from analysis and those included in the present analysis. Third, a dimorphic marker was used for linkage analysis. Although not statistically significant, the results of the linkage analysis are consistent with the findings for the association studies as indicated by the negative slope of the regression line. The failure to reach statistical significance is not surprising. Because of the limited informativity of the marker used (heterozygosity index=0.27), and the limited extent to which parental (ie, identity by descent) information was available, we had limited power to detect a statistically significant linkage. In future studies, a more informative marker should be used for linkage analysis. Finally, we used platelet aggregability to evaluate the relation between the Pl^{A} polymorphism and platelet function. Although platelet aggregation studies in platelet-rich plasma can assess the effect of platelet inhibitors such as aspirin, the in vivo correlates and clinical significance of changes in platelet aggregation need to be defined more fully.

Implications of the Study

We found that the Pl^{A2} allele was associated with increased platelet aggregability in the Framingham Offspring Study. Our results support the hypothesis that Pl^{A2} might be a genetic risk factor for CVD,[7–9] and provide a mechanism for the link. Because epinephrine-induced aggregation was increased with the Pl^{A2} allele, and because increased aggregability has been described after assumption of an upright posture[35] and strenuous exercise,[38] it would be of interest to determine whether subjects with different Pl^{A} alleles react differently to strenuous exercise. Such a study may not only provide additional insights into the mechanism by which strenuous exercise triggers the onset of cardiovascular events,[39] but may also help in selecting individuals for appropriate preventive therapy. In addition, further prospective studies are needed to test if this genetic marker is an independent risk factor for CVD. If individuals with the Pl^{A2} allele have a higher incidence of CVD, they may benefit from more aggressive measures for prevention and treatment of CVD, including therapy with antiplatelet agents such as *GP IIb/IIIa* receptor antagonists.

Acknowledgments

This study was supported by NIH/NHLBI No1-38038 to Dr. Tofler and by Research Development Award K04-HL-03138-01 from the National Heart, Lung, and Blood Institute to Dr. Lindpaintner. Linkage analysis was performed using S.A.G.E., which is supported by a USPHS Resource Grant (1P41 RR03655) from the National Center for Research Resources.

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