Increased Platelet-Collagen Interaction Associated With Double Homozygosity for Receptor Polymorphisms of Platelet GPIa and GPIIIa

Luca Pontiggia, Riitta Lassila, Stephanie Pederiva, Hans-Rudolf Schmid, Mirjam Burger, Juerg H. Beer

Objective—There is considerable controversy regarding the clinical role of the single-nucleotide polymorphisms (SNPs) of the platelet glycoprotein receptor GPIa C807T and the PlA1/A2 of GPIIIa as cardiovascular risk factors. We hypothesized that two combined SNPs in their homozygous prothrombotic forms could clarify their pathophysiological impact.

Methods and Results—We identified a family with a striking history of premature cardiovascular events and a high frequency of the prothrombotic form of the two SNPs. From this family, the platelets of a healthy, 27-year-old propositus with this double homozygosity were compared with three matched male neutral gene variant controls. The propositus had shortened PFA-100 closure times and an increased platelet aggregation response to collagen. Platelet deposition to collagen was augmented under the blood flow conditions of a high shear rate model (1600 s−1). Platelet adhesion on collagen monomers was induced in a static system, leading to the promotion of subsequent procoagulant activity.

Conclusions—The combined homozygous prothrombotic SNPs of GPIa and GPIIIa are associated with an increased platelet–collagen interaction and procoagulant activity that can be readily demonstrated in several independent systems. Our patient may serve as a useful model for the functional consequences of two combined, potentially procoagulant, platelet SNPs. (Arterioscler Thromb Vasc Biol. 2002;22:2093-2098.)

Key Words: platelet | receptor | myocardial infarction | collagen | polymorphism

The single-nucleotide polymorphisms (SNPs) of the platelet glycoprotein (GP) receptors Ia (C807T) and IIIa (PlA1/A2) have recently gained attention because they may mediate increased platelet reactivity to collagen and fibrinogen or von Willebrand factor, respectively. (For better distinction and easier understanding, the PlA2 and the GPIa 807T allelic forms of the SNPs are referred to as prothrombotic SNPs and the PlA1 and the GPIa 807C forms as neutral SNPs.) Despite a large number of well-conducted clinical trials (for GPIIIa1–3 and a list of relevant references published previously2,4 for GPIa,4–14), the results have remained controversial, in part because of the interference of multiple acquired risk factors and the polygeneity of cardiovascular disease. Different populations with varying phenotypes and end points have been studied and different settings chosen. Therefore, it is not surprising that the initially positive and sometimes spectacular results for both SNPs have been tempered by some negative trials. Even two recent large meta-analyses on the role of the PlA1/A2 SNP of GPIIIa have rendered opposite conclusions.2,3

Several lines of evidence have led us to further investigate the topic. Subgroups with a high prevalence of the prothrombotic SNPs have been identified that seem to carry an increased cardiovascular risk,15 namely, young populations with vascular disease14,16,17 and patients with restenosis after a revascularization procedure.3,18 In combination with acquired risk factors, the risk appears to increase (eg, smoking or microalbuminuria in the presence of the homozygous GPIa 807T genotype).6,8

Plausible molecular mechanisms have been found. The prothrombotic SNP of GPIa results in an increased density of this receptor and in an increased platelet adhesiveness to collagen.19–21 Interestingly, the converse appears to operate as well, because individuals with the homozygous neutral GPIa SNP (807C) with mild von Willebrand’s disease have less-effective platelet adhesion under high shear rates and perhaps more bleeding,5 and patients with congenitally low numbers of GPIa have the phenotype of a bleeding disorder.22,23

In the view of these controversies, the aim of our study was to focus on an restricted experimental human model without acquired risk factors that would meet the following 4 criteria: (1) young age, no cardiovascular disease or risk factors, (2) a positive family history for the early clinical end points, such
as stroke or myocardial infarction, (3) a high prevalence of the two prothrombotic SNPs in the family, and (4) a double homozygosity for the two prothrombotic SNPs in question.

We speculated that such an approach could offer us the opportunity to functionally analyze this potential genetic risk constellation in an unique human model.

Methods

Subjects

A white family with a striking history of multiple cardiovascular events in the age range of 27 to 50 years was analyzed. Three typical case histories are as follows:

1. A man suffered a stroke with right hemiparesis at age 27, had acute myocardial infarction at age 34, and died of a second myocardial infarction at age 35. The only risk factor was smoking.
2. A 48-year-old woman died of acute myocardial infarction without known risk factors.
3. A 38-year-old male had unstable angina and died of sudden cardiac death without known acquired risk factors.

The study was approved by the local ethics committee. Eighteen members of the family were genotyped after written informed consent was obtained. Of the three deceased family members, only one genotype was available. Our 27-year-old propositus of this family met the 4 predefined criteria. He was double homozygous for the two prothrombotic SNPs in question, and (4) a double homozygosity for the two prothrombotic SNPs in question. His blood samples revealed a normal hematologic and chemistry profile; he had a normal coagulation work-up (normal values for prothrombin time, activated partial thromboplastin time, fibrinogen, protein C, protein S, antithrombin III, factor VIIc, von Willebrand factor, homocysteine, no antiphospholipid antibodies, no prothrombin mutation [FII G20210A], and no activated protein C resistance).

Three matched male controls (ages 29, 29, 41) with the homozygous neutral SNPs were analyzed together with the propositus. The propositus was analyzed twice, and the controls were analyzed at the same time (2 controls) or 3 days apart (1 control).

Genotyping

The SNPs for GPIa C807T and GPIIIa A1/A2 were determined by polymerase chain reaction and melting point analysis with fluorescent hybridization probes by using the fluorescence energy transfer principle (LightCycler System, Roche).

Platelet Function Analyzer (PFA) and Platelet Aggregation

PFA was performed by using the platelet function analyzer PFA-100 as proposed by the manufacturer. To assess data on the kinetics of the platelet adhesion and thrombus formation, we also measured the maximal flow rate, its time point, and the time to the half-maximal deposition. Platelet aggregation was performed by using platelet-rich plasma (PRP, 250×10^6 platelets/mL) anticoagulated with 40 μmol/L HEPES, 0.1 mg/mL PPACK, 10 mmol/L HCl, and 2 mmol/L MgCl2 to provide physiological concentrations of Ca2+ and Mg2+. Platelet-rich plasma was perfused for 2 minutes at a shear rate of 1600 s⁻¹ in the Turitto’s perfusion chamber. Platelet deposition was assessed with 1 H-sorotonin labeling and by scanning electron microscopy. PRP was centrifuged from whole blood, the platelets were labeled with 10 mmol/L H-sorotonin, and reconstituted as whole blood for perfusions. This assay was performed twice in the propositus and in two controls.

Flow Cytometry

Two-hundred microliters of acidic citrate dextrose (ACD)-PRP (50×10^6 p/L/mL) were incubated with saline or 10 mmol/L thrombin receptor activator protein (TRAP-14, Sigma) and 10 μg/mL collagen type I (Sigma) for 10 minutes at room temperature with gentle stirring (100 rpm). The process was stopped with 200 μL of 1% paraformaldehyde. Ten microliters of the sample were double stained with 4 μL of CD61-FITC (anti-GPIIIa, fluorescein isothiocyanate labeled) and 2 μL of CD41-PE (anti-GPIIb, phycoerythrin identified as stroke or myocardial infarction, (3) a high prevalence of the two prothrombotic SNPs in the family, and (4) a double homozygosity for the two prothrombotic SNPs in question.

We speculated that such an approach could offer us the opportunity to functionally analyze this potential genetic risk constellation in an unique human model.
labeled) or with 2 μL of CD41-FITC and 4 μL of CD62P-PE (anti-P-Selectin) or with the respective isotype controls. After incubation for 15 minutes in the dark at room temperature, they were diluted to 2 mL with HEPES buffer. The instrument was triggered by fluorescein isothiocyanate (FITC) fluorescence and 10,000 platelets were analyzed by using the galaxy flow cytometer (DAKO Diagnostics AG). The microparticles were expressed in percent of the platelet count. This method resulted in 1% microparticles in nonactivated PRP.

To analyze the receptor density, platelets were washed once with HEPES buffer and diluted to 50×10⁶/mL. Platelets (10 to 40 µL) were labeled for 15 minutes at room temperature in the dark with CD41-FITC or the FITC-labeled isotype control (both 1:8 diluted), P1E6 (anti GPIa) or buffer and goat-anti-mouse IgG-FITC (all 1:8), polyclonal anti GPVI or preimmune serum (both 1:000), and swine-anti-rabbit IgG-FITC (1:8). All antibodies were from DAKO except for the polyclonal anti GPVI antibody, which was a kind gift of Dr. Kenneth Clemetson (Theodor Kocher Institute, University of Bern, Switzerland). Before analysis, the probes were diluted with 4 mL of HEPES buffer. The instrument was triggered by forward scatter, and 20,000 platelets were considered. The fluorescence peaks were analyzed by the FloMax-Software (DAKO).

Results

The flow cytometric data (n=3, mean±SD) indicated an increased receptor density of GPIa (+81±8%) and of GPVI (+25±4%) in the propositus compared with the control, whereas GPIIIa was remarkably stable (+3±1%).

The propositus had a shortened PFA-100 closure time compared with the mean of the three controls, namely with collagen/epinephrine (CEPI): 120 versus 163±38 s and with collagen/ADP (CADP): 78 versus 102±23 s. The maximal flow rate achieved was similar with both CADP (170 versus 177±19 µL/min) and CEPI (177 versus 163±23 µL/min). The time to the half-maximal closure was the same for CADP (54 versus 51±16 s) and slightly shortened for CEPI (89 versus 115±24 s). This suggested an accelerated platelet activation and coagulation in the second part of the measurement.

Platelet aggregation with collagen was overall increased (as illustrated in a representative tracing in Figure 1), but particularly in response to collagen monomers. The difference was maximal at critical collagen concentrations and could be overcome by increasing the dose (Figure 1). Aggregation responses to the other agonists were similar.

Under high shear rate flow conditions (1600 s⁻¹) a strongly increased platelet adhesion-induced recruitment of platelets to collagen was observed. Figure 2 represents a comparative scanning electron micrograph representing the propositus and a control under the same conditions and magnifications. The aggregates of the propositus are significantly larger and are merging via thick fibrin bundles. The control perfusion channel was evenly covered with smaller aggregates, connected by a network of pseudopods. These data are compatible with our earlier work.²⁴–²⁸–³⁰

Serotonin-positive platelets of the propositus deposited at 11.00×10⁶/cm² and those of the control at 7.21×10⁶/cm² in this example. Static platelet adhesion was enhanced in the propositus by 1.5 on collagen monomers and 1.2 on collagen fibrils (Figure 3A, left) under conditions that favored GPIa-collagen interactions in the presence of Mg²⁺ alone. Conditions that favored GPIIIa/IIIa-mediated interactions in the presence of Ca²⁺ and Mg²⁺ gave an even more prominent difference over the neutral SNPs control, namely an enhancement by 2.9 on collagen monomers and by 1.5 on fibrils (Figure 3A, right). Adhesion-triggered aggregation by higher platelet counts was not affected (Figure 3B, left), whereas the presence of both Ca²⁺ and Mg²⁺ enhanced again the difference between propositus and controls to 1.6 on monomers and 1.4 on fibrils (Figure 3B, right). The addition of Mg²⁺ alone gave overall higher adhesion, adhesion-induced aggregation as well as procoagulant activity (Figure 3, left panels). Procoagulant activity in the supernatant was enhanced accordingly (Figure 3C), thus confirming the functional relevance of the increased platelet adhesion of the propositus.

Flow cytometry analysis revealed that the platelet microparticle number of the propositus at baseline was similar to the three controls (mean±SEM): 1.7% (controls: 1.3±0.6%); however, after activation with collagen/thrombin receptor activator protein-14 (TRAP-14), it increased to a 2-fold level, namely to 101% (controls: 53±12%). In addition, more of the patient’s microparticles were positive for P-selectin: 57% (controls: 38±5%) after activation. These results are in line with the enhanced procoagulant activity.

The 18 family members that we genotyped had relatively high prevalences for the prothrombotic SNPs (Table): 11% double heterozygosity and 6% double homozygosity, 66% single heterozygosity, and 33% single homozygosity. There were only 2
members with the double homozygous-neutral SNPs. Interestingly, patient 1, who died at the age of 35 from his second myocardial infarction, was homozygous for the prothrombotic SNP of GPIa and smoked in addition, thus putting him in a high-risk situation, as shown in our earlier study.6

Discussion
The data confirm our hypothesis that the combined homozygous presence of the risk allelic forms of the two SNPs will result in an augmented and clearly detectable prothrombotic state in vitro. We have demonstrated an increased platelet reactivity in specific and elaborated tests of platelet adhesion and procoagulant activity and, interestingly, even in global tests of the laboratory routine (PFA-100, platelet aggregation). The results are in accordance with our earlier studies with heterozygous carriers of the prothrombotic SNP of GPIa (807T), which partly explain an increased reactivity to collagen in normal donors.31

Our findings of an increased platelet adhesion to collagen under static (Figure 3) and dynamic (Figure 2) conditions, an augmented aggregation with collagen (Figure 1), and an enhanced procoagulant activity (Figure 3C) associated with an increased generation of platelet microparticles, fit well into the proposed pathophysiology. On the other hand, another group32 reported that platelets isolated from Pl A2-positive donors did not show an increased ADP sensitivity, nor a PFA-100 difference, nor different binding of the Arg-Gly-Asp-peptide to platelets. This may indicate that the combined prothrombotic SNPs may be important to enhance subtle differences.

Figure 3 illustrates that in the presence of Mg2+ alone, there is an overall enhancement of platelet adhesion, aggregation, and procoagulant activity compared with Ca2+ and Mg2+.
which indicates an early inhibitory effect of Ca$^{2+}$ on the adhesive receptors.

The constellation of a double homozygosity has a very low probability, and only 1 in 750 to 1000 is affected. Therefore, the combined risk cannot be extracted from the clinical studies. The majority of the association studies have analyzed the prothrombotic SNPs in their heterozygous or both in their heterozygous and homozygous forms and vascular disease. For the $\text{PI}^{A2}$ of GPIIa, they found an odds ratios (OR) of 1 to 3, up to 6 in subgroups, and for the 807T of GPIa ORs of 2 to 4. The prevalences in the white population for $\text{PI}^{A2}$ are approximately 23% and 2%, and for GPIa 807T, 40% to 53% and 5.6 to 18%, in their heterozygous and homozygous forms, respectively. Thus, the combined heterozygosity is expected to be 9% to 12% and the combined homozygosity 0.1% to 0.4%.

The 18 family members that we genotyped had relatively high prevalences (11% double heterozygosity and 6% double homozygosity) despite the fact that only one sample of one deceased member was available. In an attempt to estimate the risk of a vascular event in a young person carrying the two homozygous prothrombotic SNPs, a conservative OR will be in the range of 4 to 10 and higher if it is more than additive. The model also will accommodate the evidence from the literature that a single acquired risk factor, such as smoking or hypertension with microalbuminuria, will substantially increase the risk in patients carrying these prothrombotic SNPs. This is well illustrated by patient 1, who died at age 35 from myocardial infarction. He had the homozygous form of the prothrombotic SNP of GPIa and an acquired risk factor (smoking) enhancing this risk.

These patients should reduce their acquired risk factors and perhaps consider a prophylactic antiplatelet regimen. Interestingly, however, the antithrombotic effect of aspirin seems to be reduced in $\text{PI}^{A2}$ platelets.

Thus, the physiological sequence of platelet-vessel wall interactions in hemostasis helps to explain the combined prothrombotic effect of the SNPs (hypothesis illustrated in Figure 4). After GPIb-dependent platelet rolling and tethering to the subendothelium, the platelet monolayer develops on the subendothelial matrix mediated by GPIa/Ia and GPVI as collagen receptors. Platelets lacking GPIa may not be able to activate integrins and fail to fully adhere and aggregate on collagen; then, the platelet-rich thrombus is generated by the interaction of GPIIb/IIIa with fibrinogen and/or von Willebrand factor. Based on these mechanisms, our propositus may have an amplifying effect at the following three levels: (1) a higher, genetically determined receptor density of GPIa will result in an increased adhesion to collagen, (2) this is enhanced by an increased density of GPVI (which is correlated with higher GPIa-levels), with a gain of function and an enhanced procoagulant response, and (3) the haplotype $\text{PI}^{A2}$ of GPIIIa increases and stabilizes the interaction of GPIIb/IIIa with immobilized fibrinogen, and it enhances the platelet aggregability and the thrombin generation.

Although we were careful to screen for the known congenital or acquired risk factors, we cannot exclude additional prothrombotic SNPs or pathologies that might contribute to the results.

In conclusion, our analysis fulfills the three generally accepted requirements for the polymorphism-disease association: a plausible mechanism (the receptor density and the gain of function), the functional consequence (platelet-adhesion, platelet-aggregation, procoagulant activity), and the association with disease.

**Acknowledgments**

This work was supported by the Swiss National Science Foundation by Grant-No. 32-59449.99 (to J.H.B.) and the Swiss Heart Foundation (to J.H.B.). We thank T. Järvenpää and M. Lemponen for outstanding technical assistance and L. Jeangros and K. Zehnder for secretarial support.
Increased Platelet-Collagen Interaction Associated With Double Homozygosity for Receptor Polymorphisms of Platelet GPIa and GPIIIa
Luca Pontiggia, Riitta Lassila, Stephanie Pederiva, Hans-Rudolf Schmid, Mirjam Burger and Juerg H. Beer

*Arterioscler Thromb Vasc Biol.* 2002;22:2093-2098; originally published online October 17, 2002;
doi: 10.1161/01.ATV.0000042230.26207.D2

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/22/12/2093