The number of platelet glycoprotein Ia molecules is associated with the genetically linked 807 C/T and HPA-5 polymorphisms

J. Corral, J. Rivera, R. González-Conejero, and V. Vicente

BACKGROUND: The neutral 807 C/T (Phe²⁹⁰) polymorphism (807 C/T polymorphism) of the glycoprotein (GP)Ia gene has been recently associated with the number of GPIa molecules on the platelet surface. The association of the number of GPIa molecules with other GPIa polymorphisms, such as HPA-5 (Glu/Lys⁹⁹⁰) (HPA-5 polymorphism), involved in alloimmune thrombocytopenias is unknown.

STUDY DESIGN AND METHODS: The association of the HPA-5 polymorphism with the number of GPIa molecules on the platelet surface in 159 white blood donors was investigated. The genetic linkage between the HPA-5 and the 807 C/T polymorphisms in 316 individuals was also determined.

RESULTS: Both the 807 C/T and HPA-5 polymorphisms correlate with the number of GPIa molecules on the platelet surface. The 807 T and HPA-5b alleles are associated with increased numbers of GPIa molecules on the platelet surface. Moreover, the HPA-5b allele is genetically linked to 15.8 percent of the 807 C alleles. Therefore, the number of GPIa molecules on the platelet surface is dependent on both GPIa polymorphisms as follows: 807 T/T, HPA-5 a/a > 807 C/T, HPA-5 a/b > 807 C/C, HPA-5 a/a > 807 C/C, HPA-5 a/b > 807 C/C, HPA-5 a/a.

CONCLUSION: Two GPIa polymorphisms (807 C/T and HPA-5) responsible for the variability in the numbers of GPIa/Ila molecules on the platelet surface in whites have been identified. Despite the genetic linkage between the two polymorphisms, their influence on the number of GPIa molecules on the platelet surface may occur through different mechanisms.

The glycoprotein Ia (GPIa), also known as αβ integrin and very late antigen 2, is a member of the integrin family that acts as a major receptor for collagen in human platelets and in other cell types.¹⁻⁵ Through its role as a collagen receptor, GPIa has the potential to contribute significantly to platelet function and hemostasis in vivo. Patients with a deficiency of the collagen receptor show a tendency to bleed,⁶⁻⁸ and patients with serum anti-GPIa show platelet dysfunction in adhesion or aggregation.⁹⁻¹⁰

In comparison with other platelet receptors such as GPIb/IX/V and GPIIb/IIIa, GPIa is present on the platelet membrane in much smaller numbers.¹¹⁻¹³ Moreover, while the number of GPIb/IX/V and GPIIb/IIIa molecules on the platelet surface shows little variation between individuals, the number of GPIa/Ila molecules on the platelet surface has been reported to vary by as much as an order of magnitude among randomly selected subjects.¹³ Recently, Kunicki et al.¹⁴ reported a potential genetic marker of similar variability. These investigators identified two linked conservative polymorphisms within the coding sequence of the GPIa gene—at codon Phe²⁹⁰ (807 C/T polymorphism) and ACA/ACG at codon Thr²⁴⁶ (873 G/A polymorphism)—that correlated with the number of GPIa molecules on platelets in United States whites.¹⁴ The alleles (TTC and ACG) corre-

ABBREVIATIONS: GP = glycoprotein; MAIPA = monoclonal antibody-specific immobilization of platelet antigens; MFI = mean fluorescence intensity; NAT = neonatal alloimmune thrombocytopenia; PCR = polymerase chain reaction; PRP = platelet-rich plasma.

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lated significantly with decreased numbers of GPIa molecules on the platelet surface, whereas the complementary alleles (TTT and ACA) were associated with increased numbers of these molecules on the platelet surface. Other mutations had been described previously in the GPIa gene, in addition to the 807 C/T and 873 G/A polymorphisms. Among those, the most extensively investigated is the A/G polymorphism at position 1648, leading to a change from Lys (AAG) to Gln (GAG) at amino acid residue 505 (HPA-5 polymorphism). This single amino acid polymorphism is of clinical relevance because it accounts for the biallelic HPA-5 system, also known as Zav, Br, or Hc. The HPA-5a allele contains a glutamic acid at position 505, whereas the HPA-5b allele has a lysine at that location. In whites, the frequencies of these alleles are 90 and 10 percent for HPA-5a and HPA-5b, respectively. Alleloimmunization to antigens in the HPA-5 system has been described as the second most common cause of neonatal alloimmune thrombocytopenia (NAIT) in whites, and anti-HPA-5b might also be involved in refractoriness to platelet transfusion and posttransfusion purpura.

Kiefel et al. use of the monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assays and with an indirect competitive enzyme-linked immunosorbent assay, first noted a quantitatively higher immunoreactivity of anti-HPA-5b serum with human platelets of the HPA-5 b/b genotype than that noted with platelets of the HPA-5 a/b genotype. However, the potential correlation of the HPA-5 polymorphism with the number of GPIa molecules on the platelet surface has scarcely been investigated.

To provide further insight into this issue, we have evaluated the number of GPIa molecules on platelets from 159 Spanish white individuals by direct flow cytometry and binding assays with specific GPIa monoclonal antibodies. These subjects were also genotyped for the HPA-5 and 807 C/T polymorphisms, and the potential correlation of either polymorphism with the number of GPIa molecules on the platelet surface was assessed. In this report, we also provide data for 316 selected individuals that suggest a genetic linkage between the HPA-5 and the 807 C/T polymorphisms. Finally, we investigated the number of GPIa molecules on the platelet surface in a cohort of neonates, to determine if the number of GPIa molecules on the platelet surface in newborns facilitates the occurrence of NAIT.

MATERIALS AND METHODS

Subjects
Measurement of the number of GPIa molecules on the platelet surface and genetic analysis of the HPA-5 and 807 C/T polymorphisms, were carried out in 159 unrelated and randomly selected white blood donors (79 men and 80 women; mean age, 36.2 ± 11.0 years) from Murcia (southern Spain). Similar studies were also performed in a group of 31 randomly selected neonates from whom umbilical cord blood samples were obtained at birth. The potential linkage between the HPA-5 and 807 C/T polymorphisms was investigated in a group of 316 selected whites (108 with the 807 C/C polymorphism, 106 with the 807 C/T polymorphism, and 102 with the 807 T/T polymorphism). This work had the approval of the local ethics committee and was performed according to the Declaration of Helsinki as amended in Venice in 1983.

DNA extraction
Total genomic DNA was obtained from white cells after lysis with sodium dodecyl sulfate and proteinase K treatment. DNA samples were purified by the use of phenol/chloroform and ethanol precipitation and stored at −80°C until use.

Genotyping of exon and intron 7 of GPIa
Genomic polymerase chain reaction (PCR) of the GPIa exon and intron 7 was performed essentially as described, using two oligonucleotide primers: reverse primer, 5'gatttaactctcccagctgcctc-3' and the mutantagen forward primer, 5'-atggtgggacacccacacagtt-3' (corresponding to nucleotides 173-196 of the intron sequence and 782-806 of the exon 7 sequence respectively); nucleotide numbers are according to Takada and Helmer. The mutated nucleotide (bold italic) in the forward primer allows the identification of the 807 C/T polymorphism of the GPIa gene by restriction of the PCR product (3 μL) with 1 U of Hinf I (New England Biolabs, Beverly, MA) at 37°C for 3 hours. The restriction pattern was analyzed in 5-percent acrylamide gel, electrophoresed at 300 V for 15 minutes, and stained with AgNO₃ as reported. The 807 Callele of GPIa corresponded to a band pattern after Hinf I restriction of 209 bp, whereas the presence of a 231-bp band was distinctive of the 807 T allele (Fig. 1A).

Genotyping of HPA-5
Two oligonucleotide primers, HPA-5F and HPA-5B, were used in a PCR amplification (modified from elsewhere) to generate a 274-bp fragment of the GPIa gene containing the sequence responsible for HPA-5a and HPA-5b. The PCR product (3 μL) was digested with 1 unit of Mnl I (New England Biolabs) at 37°C for 3 hours, and the restriction pattern was analyzed in acrylamide gels stained as above. The HPA-5a allele displayed a pattern of four bands, of 136, 97, 33, and 8 bp, whereas the presence of three bands (169, 97, and 8 bp) was distinctive of the HPA-5b allele (Fig. 1B).

Flow cytometric analysis of the platelet collagen receptor GPIa
The expression of GPIa, GPIIIa, and GPIV on the platelet surface was analyzed in 159 blood donors and in 31 healthy newborns following platelet immunofluorescence staining.
with the specific monoclonal antibodies: GPIa (CD49b* fluorescein isothiocyanate [FITC], clone 10G11, PeliCluster, Amsterdam, Netherlands), GPIIIa (CD61*FITC, Becton Dickinson, San Jose, CA), and GPIP (CD36, clone IVC7, PeliCluster). Parallel immunostaining with an irrelevant isotype murine IgG*FITC (Becton Dickinson) served as the negative control. These assays were performed in diluted platelet-rich plasma (PRP) (50,000 cells/μL) as detailed elsewhere.34 Fluorescence flow cytometric analysis was performed with a flow cytometer (FACScan, Becton Dickinson). For each sample, data acquisition of 5000 events was gated on forward and side-angle light scatter with gains adjusted to include the platelet population. Fluorescence of the stained platelets was analyzed with software (Cell Quest, Becton Dickinson) to obtain both the percentage of positively stained cells and their mean fluorescence intensity (MFI).

Quantitative assessment of the number of GPIa molecules on the platelet surface by binding assays

In selected samples from subjects carrying different 807 C/T and HPA-5 a/b genotypes, a determination of the number of GPIa molecules on the platelet surface was achieved by binding experiments with a GPIa monoclonal antibody (PIE6, CAMFolio, Becton Dickinson), previously radiolabeled (specific activity, 133 Ci/mmol) with carrier-free Na-125I (Amersham Ibérica SA, Madrid, Spain) using Iodogen (Pierce Chemical, Rockford, IL).35 The hot binding assays were carried out in diluted PRP (100,000 platelets/μL) as described,36 and the binding isotherms were analyzed with the computer-assisted program (LIGAND, Elsevier-Biosoft, Cambridge, UK) to obtain the binding parameters.37

Statistical analysis

All values are reported as mean ± SD. Changes in variables between groups of subjects with different genotypes were tested by the t-test. Differences were considered significant when p<0.05.

RESULTS

Genetic distribution of 807 C/T and HPA-5 polymorphisms of GPIa gene

The genotype and allelic frequencies for the 807 C/T and HPA-5 polymorphisms in the normal population from southern Spain were derived from genetic analysis of 159 randomly selected blood donors. As shown in Table 1, for the donors with the 807 C/T polymorphism, we found that 43.4 percent were C/C, 44.0 percent were C/T, and 12.6 percent were T/T. Thus, the C allele was present at a frequency of 0.65, whereas the allelic frequency for the T allele was 0.35 (Table 1). Of the 159 blood donors, 133 were found to be homozygous for HPA-5a (HPA-5a/a), while 26 individuals were carriers of the HPA-5b allele (HPA-5a/b or HPA-5b/b) (Table 1). The corresponding allelic frequencies were 0.915 and 0.085 for HPA-5a and HPA-5b, respectively. These results are in agreement with the genotypic and allelic frequencies for the 807 C/T and HPA-5 polymorphisms, previously described in European and US whites.14,17-20

Association of the number of molecules of GPIa on the platelet surface with the 807 C/T and HPA-5 polymorphisms

To clarify the potential association of the 807 C/T polymorphism and/or the HPA-5 polymorphism with the number of GPIa molecules on the platelet surface, we performed direct flow cytometric analysis of the number of GPIa molecules on the platelet surface in 159 randomly selected blood donors by staining with a CD49b FITC-labeled monoclonal antibody. Table 1 summarizes the results obtained in this study, showing the MFI according to the 807 C/T and HPA-5 genotypes. We found an obvious association of both polymorphisms with the number of molecules of GPIa on the platelet surface. As shown, platelets from T/T carriers displayed significantly higher MFI than platelets from C/T.

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<th>TABLE 1. Expression of GPIa, GPIIIa, and GPIP according to the 807 C/T and HPA-5 genotypes</th>
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<td>Genotype</td>
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* The MFI of CD49b was significantly different (p<0.001) for C/C vs. C/T, C/C vs. T/T, and C/T vs. T/T subjects.
† The MFI of CD49b in HPA-5 a/a subjects and carriers of the HPA-5b allele (HPA-5 a/b and b/b) was significantly different (p = 0.037).
individuals and C/C subjects. Significant differences were also observed between C/T and C/C carriers (Table 1). In addition, we detected a significant association of the HPA-5b allele with increased numbers of GPIa molecules on the platelet surface; conversely, decreased numbers were associated with the HPA-5a allele (Table 1). When we analyzed the influence of the number of GPIa molecules on the platelet surface when the two polymorphisms were present together, we found that the presence of the HPA-5b allele significantly increased the number of GPIa molecules in both C/C and C/T subjects. Thus, C/C subjects carrying the HPA-5b allele demonstrated significantly higher numbers of GPIa molecules on the platelet surface than C/C individuals who were homozygous for HPA-5a (HPA-Sala). Similar results were found among C/T subjects (Table 2). In this study, we found no differences in the number of GPIa molecules on the platelet surface, or in its association with the 807 C/T and HPA-5 polymorphisms, in relation with the age or sex of the individuals.

To confirm these results, quantitative determination of the number of GPIa molecules on the platelet surface by means of binding assays with the 125I-labeled monoclonal anti-GPIa (125I-P1E6) was performed in subjects with distinct combinations of 807 C/T and HPA-5 genotypes. As illustrated in Fig. 2, the results of these assays, in which we used a different monoclonal anti-GPIa than in flow cytometric studies, further revealed the association of the 807 C/T and HPA-5 polymorphisms with the number of GPIa molecules on the platelet surface. Thus, the number of GPIa molecules on the platelet surface according to genotypes was 807 T/T, HPA-5 a/a > 807 C/T, HPA-5 a/a > 807 C/C, HPA-5 a/b > 807 C/C, HPA-5 a/a.

Finally, the flow cytometric studies conducted in the cohort of 31 randomly selected neonates showed a similar interindividual variation in the number of GPIa molecules on the platelet surface as that found in adults (MFI for CD49b-positive platelets, 11.17 ± 4.0 [newborns] vs. 11.25 ± 4.4 [adults], p=0.92). Moreover, in these neonates, the association of the number of GPIa molecules on the platelet surface with the 807 C/T and HPA-5 polymorphisms was identical to that observed in the group of blood donors (data not shown).

As expected, in the current study we found no association of either the 807 C/T polymorphism or the HPA-5 polymorphism of the GPIa gene with the number of molecules on the platelet surface of other collagen receptors: GPIIa (CD61) or GPIV (CD36) (Tables 1 and 2).

**Linkage of the 807 C/T and HPA-5 polymorphisms of GPIa gene**

The results obtained in the studies described above, showing no HPA-5b allele among 20 T/T subjects, prompted us to investigate whether there was a genetic linkage between the 807 C/T and HPA-5 polymorphisms of the GPIa gene. For that purpose, we selected 318 white subjects previously genotyped for the 807 C/T polymorphism—108 C/C, 106...
CT, and 102 T/T. As presented in Table 3, we found that the 807 T allele was always linked with the HPA-5a allele, whereas 15.8% percent of the 807 C alleles were associated with the HPA-5b allele. Therefore, these two polymorphisms describe three alleles: 807 C, HPA-5a, 807 C, HPA-5b, and 807 T, HPA-5a.

DISCUSSION

GPIa, widely distributed in a variety of tissues, is expressed on megakaryocytes and platelets. It is present on the platelet surface in smaller numbers than are other platelet receptors such as the GP Ibα/IX/V and GP IIb/IIIa. Moreover, the number of molecules of GPIa/IIa on the surface of human platelets has previously been reported to differ by a factor of 10. The present study, in which the number of GPIa molecules was investigated in platelets from 159 randomly selected blood donors, further confirms the heterogeneity in the number of GPIa molecules on the platelet surface among randomly selected white individuals.

We evaluated the number of molecules of GPIa on the platelet surface by flow cytometric analysis and binding assays, using GPIa monoclonal antibodies distinct from those previously employed by others. This heterogeneity could have a physiologic relevance, as it is well established that GPIa is a major receptor mediating platelet adhesion and responsiveness to collagen. Additionally, we found that the degree of variation in the number of molecules of GPIa on the platelet surface is similar in adults and neonates, which suggests that the number of GPIa molecules on the platelet surface is not a key factor for the high incidence of NAIT associated with HPA-5b. Therefore, the reported deficiency in collagen-induced activation of newborn platelets is unlikely to result from a lower number of GPIa molecules.

The precise genetic basis for the observed heterogeneity remains to be elucidated. Most likely, genetic alteration(s) within regions of the GPIa gene that control either transcription rate or stability and turnover of mRNA would be responsible for such variability in the numbers of GPIa molecules on the platelet surface, but so far, only silent polymorphisms and the Glu585/Lys585 amino acid polymorphism (the HPA-5 polymorphism) have been identified within the coding sequence of the GPIa gene. We confirmed and extend the observation of Kunicki et al. that the conservative biallelic polymorphism 807 C/T, which is in linkage disequilibrium with the 873 G/A polymorphism, correlates with the number of GPIa molecules on the platelet surface (Table 1 and Fig. 2). Furthermore, our study also demonstrated an association of the HPA-5 polymorphism with the number of molecules of GPIa. Our finding that the HPA-5b allele is associated with higher numbers of GPIa molecules is in agreement with the indirect observations previously reported by Kiefel et al. However, our results are in contrast with the absence of correlation between HPA-5 phenotype and the number of GPIa molecules found by Kunicki et al. in only nine subjects. Because our data show that increased numbers of GPIa molecules on the platelet surface were associated with both the 807 T and HPA-5b alleles, a genetic linkage between these alleles might have been expected. However, we detected the opposite linkage. In other words, the HPA-5b allele (correlating in our study with higher numbers of GPIa molecules) was genetically linked to the 807 C allele (associated with lower numbers of GPIa molecules). This genetic linkage results in the number of GPIa molecules in the genotype HPA-5 a/b, 807 C/C being one-half the number in the HPA-5 a/b, 807 C/T genotype (MFI, 7.7 vs. 14.5, respectively) (Table 2). Therefore, the mechanism responsible for the differing numbers of GPIa molecules associated with the 807 C/T and HPA-5 polymorphisms should be different. According to our results and as suggested by others, the 807 C/T polymorphism should be linked to genetic alteration(s) located in regulatory regions of the GPIa gene. Consequently, the HPA-5 polymorphism might be linked to a different genetic variation that is able to influence the gene at the transcription level; alternatively, the Glu585/Lys585 amino acid change (HPA-5 polymorphism) could affect the stability of the protein or the formation of the GPIa/IIa complex, thus determining the number of GPIa/IIa complexes.

Even though a low number of GPIa molecules are present on the platelet surface, the genetically determined level of this receptor might have an impact on platelet function and adhesion to collagen, thus influencing long-term hemostasis, especially in individuals exposed to situations of increased hemostatic risk. The number of GPIa molecules on the platelet surface would also affect the platelet immunogenic capacity to trigger the production of anti-HPA-5a and/or anti-HPA-5b. Finally, the role of antibodies that react with platelet glycoproteins in thrombocytopenia could be related to the antigenic dose of the targeted protein on the platelet surface. The high variability in the number of GPIa molecules on the platelet surface, and the genetic mechanisms responsible for such variability, could also help to

| TABLE 3. Genetic linkage between 807 C/T and HPA-5 polymorphisms of the GPIa gene |
|---------------------------------|------|-------|-------|----------------|----------------|
| 807 C/T Genotype               | b/b  | a/b   | a/a   | a   | b   |
| Number (%)                    | Number (%) | Number (%) | a/b | 0.838* | 0.162* |
| C/C                            | 78 (70.4) | 29 (26.9) | 3 (2.8) | 0.925* | 0.076* |
| C/T                            | 90 (84.9) | 16 (15.1) | 0     | 1.000* | 0* |
| T/T                            | 102 (100) | 0     | 0     | 1.000* | 0* |

* Allele frequencies.

376 TRANSFUSION Volume 39, April 1999
clarify the discrepancy between the high prevalence of anti-HPA-5b in fetomaternal mismatches and the low incidence of NAIT associated with anti-HPA-5b. For all these situations, the identification of the genetic markers that determine the number of GPIa molecules on the platelet surface could be useful.

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REFERENCES

Volume 39, April 1999 TRANSFUSION 377


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