Role of the 807 C/T Polymorphism of the $\alpha_2$ Gene in Platelet GP Ia Collagen Receptor Expression and Function

Effect in Thromboembolic Diseases

J. Corral, R. González-Conejero, J. Rivera, F. Ortuño, P. Aparicio, V. Vicente

From the Unit of Onco-Hematology and Hemotherapy, and 1Biochemistry Department, School of Medicine, Hospital General Universitario, Murcia, Spain

Summary

The variability of the platelet GP Ia/Iia density has been associated with the 807 C/T polymorphism (Phe 224) of the GP Ia gene in American Caucasian population. We have investigated the genotype and allelic frequencies of this polymorphism in Spanish Caucasians. The T allele was found in 35% of the 284 blood donors analyzed. We confirmed in 159 healthy subjects a significant association between the 807 C/T polymorphism and the platelet GP Ia density. The T allele correlated with high number of GP Ia molecules on platelet surface. In addition, we observed a similar association of this polymorphism with the expression of this protein in other blood cell types. The platelet responsiveness to collagen was determined by "in vitro" analysis of the platelet activation and aggregation response. We found no significant differences in these functional platelet parameters according to the 807 C/T genotype. Finally, results from 3 case/control studies involving 302 consecutive patients (101 with coronary heart disease, 104 with cerebrovascular disease and 97 with deep venous thrombosis) determined that the 807 C/T polymorphism of the GP Ia gene does not represent a risk factor for arterial or venous thrombosis.

Introduction

Collagen plays an important role in platelet adhesion to the exposed subendothelium. Moreover, collagen is a remarkable ligand for platelets since it causes platelet activation and aggregate formation (1). Although several glycoproteins have been proposed to mediate platelet adhesion to subendothelium, the most extensively studied and best documented receptor for collagen on platelets is the glycoprotein Ia/Iia (GP Ia/Iia) (GP Ia/Iia) (1, 2). This receptor, also called $\alpha_\beta_1$ integrin and very late antigen 2 (VLA-2), is widely distributed on various cell types including endothelial cells, fibroblasts, and epithelial cells from multiple sites (3). The key role of the GP Ia/Iia receptor in platelet function and hemostasis is revealed by the bleeding tendency shown by patients with deficiency for this receptor (4-6). Moreover, platelet dysfunction in adhesion or aggregation is observed as a consequence of anti-GP Ia serum autoantibodies (7, 8).

A few years ago, it was documented a high variability in the density of GP Ia/Iia on human platelets from randomly selected individuals (9). A genetic marker of such variability has been very recently identified in the GP Ia gene (10). Two linked neutral polymorphisms TTT/TTC (Phe 224) and ACA/ACG (Thr 246) were found statistically associated with the platelet GP Ia density in American Caucasian population. The allele TTC...ACG correlated with low levels of GP Ia on the platelet surface, whereas the complementary allele (TTT...ACA) was associated with high levels of this receptor. According to the role of platelet adhesion in hemostasis and thrombosis, and to the probable synergy between environmental and genetic factors to determine the overall risk for thrombosis in any individual, a high density of a collagen receptor could represent a potential risk factor for thrombotic diseases.

In this study we have determined the frequency of the recently described 807 C/T polymorphism of the GP Ia gene in Spanish Caucasian individuals. Moreover, we evaluated further the association of this polymorphism with the GP Ia expression on platelets, monocytes and activated T cells. Finally, we assessed the role of this polymorphism in platelet activation induced by several agonists, and its putative importance as a genetic risk factor for thrombotic disorders.

Subjects, Materials and Methods

Subjects

The study involved 101 consecutive patients diagnosed of coronary heart disease (CHD) according to the World Health Organisation criteria (11), who survived a primary acute coronary event at the Hospital Coronary Unit. In addition, we investigated 104 consecutive patients with ischaemic cerebrovascular disease (CVD) referred to our institution. All CVD patients underwent brain computed tomography or MRI. Diagnosis of CVD was attained according to the classification of cerebrovascular diseases of the National Institute of Neurological Disorders and Stroke ad Hoc Committee (12). All CVD patients included in our study presented cerebral ischaemia, excluding those patients with cerebral haemorrhage. Finally, 97 consecutive patients with a confirmed diagnosis (by compression ultrasonography or contrast venography) of deep venous thrombosis (DVT) were also evaluated. All included cases of CHD, CVD or DVT were age, sex matched to a different control. Most, if not all thrombotic disorders are multifactorial diseases in which different risk factors trigger the thrombus formation. We assume that a single genetic polymorphism by itself, could never be responsible of a thrombus, but it could influence the risk to develop the disease in association with other factors. Therefore, our strategy for identification of polymorphisms predisposing to the disease was to avoid overrepresentation of classic vascular risk factors in the CVD and CHD groups compared to controls. Thus, we tried to approximate risk factors between every case patient and a single control who also matches selected risk factors for arterial thrombotic disease (smoking history, blood pressure, total serum cholesterol level and diabetes status) with his/her respective case patient. These controls were selected among a population of patients admitted to the hospital who had no documented history of
vascular disease as determined by a complete questionnaire in a personal interview with all candidates, and review of his/her clinical history.

Finally, to determine the frequency of the GP 1a polymorphism in the population from our region, we performed genotype analysis in additional 284 unrelated and unselected Caucasian healthy blood donors (Table 1).

All subjects investigated gave their informed consent to enter the study, which had been approved by the local ethics committee.

Blood Sampling

Blood samples were collected by venipuncture into trisodium citrate (1/10). Platelet-rich plasma (PRP) and platelet-poor plasma were obtained by centrifugation. Washed platelets were prepared by the procedure of Mustard (13). DNA was extracted as described (14).

Genotyping of the 807 C/T Polymorphism

Genomic polymerase chain reaction (PCR) of the GP 1a exon/intron 7 was performed using two oligonucleotide primers: 5' gattacttttctcagctctc3' [reported by Kunicki et al. (10)] and the mutagenic 5' aggggaggagcacaacaaga3' (corresponding to nucleotides 173-196 of the exon sequence, and 782-806 of the exon 7 sequence, respectively) [nucleotide number according to Takada et al. (15)]. The mutated nucleotide in the forward primer (bold underlined) allowed the identification of the 807 C/T (Phe 224) polymorphism of the GP 1a gene by restriction of the PCR product with Hinf I (New England Biolabs, Beverly, MA), followed by electrophoresis in acrylamide gels stained with AgNO3 (16). The 807 C allele of the GP 1a gene displayed a band pattern of 209 base pairs (bp), whereas the presence of a 231 bp band was distinctive of the 807 T allele (Fig. 1).

Flow Cytometric Analysis on Platelets

The platelet surface expression of GP 1a, GP IIIa and GP IV was estimated following immunofluorescence staining with specific monoclonal antibodies (MoAbs): anti-GP 1a CD49b*FITC (clone 10G11 from PeliCluster, Amsterdam, Netherlands), anti-GP IIIa CD61*FITC (Becton Dickinson, San Jose, CA) and anti-GP IV CD36 (clone 1VC7 from PeliCluster). Parallel immunostaining with an irrelevant isotype murine IgG*FITC (Becton Dickinson) served as negative control. These assays were performed in diluted PRP essentially as detailed elsewhere (17). The percentage of positively stained cells, and the mean fluorescence intensity (MFI) were analyzed with the CellQuest™ software (Becton Dickinson).

Binding Assays

In selected samples from subjects carrying different 807 C/T genotype, a quantitative determination of the platelet number of GP 1a molecules was achieved by binding experiments with iodine labelled anti-GP 1a monoclonal antibody PIE5 (CAMFollo, Becton Dickinson) (18). The hot binding assays were carried out in diluted PRP (100,000 platelets/μl) suspensions as described (19), and the binding isotherms were analyzed with the computer-assisted program LIGAND (20).

Expression of the GP 1a in other Cells

We assessed the surface expression of the GP 1a on monocytes and activated T lymphocytes from healthy individuals representative of each 807 C/T genotypes (C/C, C/T and T/T).

For monocytes, triple immunostaining of cells was achieved in blood samples mainly as reported (21), using the Mo Abs CD49b* FITC (clone 10G11, PeliCluster), CD 14*PE (clone M09P, Becton Dickinson) and HLA-DR*PerCP (clone L243, Becton Dickinson). Negative controls with irrelevant isotype antibodies were run in parallel. Fluorescence flow cytometric analysis was performed using a FACSCalibur instrument (Becton Dickinson). For each sample run, data acquisition of 50,000 events was performed using the CellQuest™ software. Linear values (1-10,000) were used as log data units. The fluorescence of stained monocytes was evaluated by a two-step analysis using both the CellQuest™ and the Paint-A-Gate Pro™ Softwares (Becton Dickinson).
Table 2: Genomic and allelic frequencies of the 807 C/T polymorphism of the GP Ia in healthy subjects and in the case/control studies

<table>
<thead>
<tr>
<th>GENOTYPE</th>
<th>CHD</th>
<th>CVD</th>
<th>DVT</th>
<th>Blood donors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Case patients</td>
<td>% Controls</td>
<td>P*</td>
<td>% Case patients</td>
</tr>
<tr>
<td></td>
<td>(n = 101)</td>
<td>(n = 101)</td>
<td></td>
<td>(n = 104)</td>
</tr>
<tr>
<td>C/C</td>
<td>44.6</td>
<td>39.6</td>
<td>0.934</td>
<td>35.6</td>
</tr>
<tr>
<td>C/T</td>
<td>45.5</td>
<td>42.6</td>
<td>0.671</td>
<td>46.2</td>
</tr>
<tr>
<td>T/T</td>
<td>9.9</td>
<td>17.8</td>
<td>0.103</td>
<td>18.3</td>
</tr>
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</table>

*Chi-square test was used to compare the values of a particular GP Ia genotype among case patients and controls.
† Chi-square test was used to compare the values of the distribution of GP Ia genotypes/haplotypes among case patients and controls.
Abbreviations: CHD, coronary heart disease; CVD, cerebrovascular disease; DVT, deep venous thrombosis.

Firstly, CD14+ or CD14+/HLA-DR+ cells with intermediate FSC and SSC were gated, and recorded as new files. Then, the CD49b*FITC fluorescence was analyzed in those events. Peripheral blood lymphocytes (PBL) from selected donors were isolated by Ficoll-Hypaque density gradient centrifugation (Sigma-Aldrich, Madrid, Spain). Lymphoblastoid cell lines GUS and R69 (EBV-transformed normal B cells) were grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-Glutamine and 1% penicillin-streptomycin (complete medium CM) (GIBCO-BRL, Barcelona, Spain). T lymphocytes were expanded by seeding 10⁶ PBL in CM supplemented with 1% heat-inactivated pooled human serum from A+AB male donor, and 20 U/ml recombinant IL-2 (T cell medium) (GIBCO-BRL) in round bottom 96-well plates containing 2 x 10⁶ irradiated mycoplasma free EBV-B cell lines (60 Gy). Growing cells were restimulated weekly by seeding 1-2 x 10⁶ activated T lymphocytes in round bottom 96-well plates containing 2 x 10⁶ irradiated allogeneic PBL and 2 x 10⁶ irradiated lymphoblastoid B cell lines (LCL) in 200 μl of T cell medium as described (22). Every week, activated bulk cultures were analyzed by immunofluorescence after staining with anti-CD3*PE (Caltag, Burlingame, CA) and anti-CD49b*FITC (clone 10G11). The fluorescence of stained T cells was analyzed as described for platelets to obtain both the percentage of positively stained cells, and the mean fluorescence intensity.

In Vitro Platelet Functional Studies

The putative influence of 807 C/T genotype in platelet reactivity was investigated in parallel with samples from individuals representative of each genotype (C/C, C/T and T/T) matched for age, race and sex. First, we assessed the aggregation profiles of washed platelets (250,000/well), stimulated with either heparin buffer (Sigma-Aldrich), thrombin (1U/ml) (specific clotting activity 5459 NIH/mg of protein; Calbiochem-Novabiochem AG, Lucerne, Switzerland), and calf skin type I collagen (40, 20, and 10 μg/ml) (Sigma-Aldrich). Changes in light transmission due to platelet aggregation were recorded for a total time of 10 min using an Aggrecorder II aggregometer (Menarini Diagnostics, Florence, Italy).

The influence of the 807 C/T genotype in platelet activation pattern was evaluated in PRP samples under both static and stirring conditions. For static conditions, diluted PRP (6000 cells/μl) was incubated (30 min, RT) with heparin buffer, type I collagen (800, 400, and 200 μg/ml) or thrombin (1 NIH U/ml). The fibrin formation inhibitor peptide GPRP (2.5 mM) (Sigma-Aldrich) was included to avoid thrombin induced clotting. Dynamic platelet activation was achieved by incubating PRP (250,000 platelets/μl) with heparin buffer, ADP (10 μM) (Sigma-Aldrich), and type I collagen (200 μg/ml) for 5 min at 37° C at 1000 rpm in the Aggrecorder II aggregometer. To avoid agonist-induced aggregation, the PRP samples were previously incubated (30 min, RT) with the anti-GP IIb/IIIa monoclonal antibody LJ-CP8 (0.14 mg/ml). LJ-CP8, kindly provided by Dr. Z. M. Rutger (Research Institute of Scripps Clinics, CA, USA), is a murine IgG1 that recognises the complex IIb/IIIa, and blocks its binding to vWF and fibrinogen (23).

Platelet activation, both under static or stirring conditions, was evaluated by flow cytometric analysis of the surface expression of the α-granule associated glycoprotein P-selectin (CD62) and granulophysine (CD63). These analyses were performed as described above, using the specific Mo Abs CD62*PE (Becton Dickinson) and CD63*FITC (Immunotech, Marseille, France).

Statistical Analysis

All numerical variables are given as mean ± standard deviation (SD). Changes in variables between patient and control groups, and between subjects with different genotype, were tested by the Student's t-test or the chi-square test as appropriate. Differences were considered significant when p ≤ 0.05.

Table 3: Surface expression of GP Ia, GP IIa and GP IV in platelets, monocytes and activated T cells

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Platelets</th>
<th>Monocytes</th>
<th>Activated T cells</th>
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<tr>
<td></td>
<td>N</td>
<td>GP Ia</td>
<td>GP IIa</td>
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<tr>
<td>C/C</td>
<td>69</td>
<td>8.71 ± 2.59%</td>
<td>288 ± 52.9</td>
</tr>
<tr>
<td>C/T</td>
<td>70</td>
<td>12.39 ± 3.94%</td>
<td>294 ± 71.9</td>
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<tr>
<td>T/T</td>
<td>20</td>
<td>15.89 ± 4.51%</td>
<td>312 ± 44.4</td>
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Values resulted from double measurements from each individual and represent mean fluorescence intensity (MFI) ± standard deviation. 
† Values correspond to MFI of CD 49b at day +2 of stimulation.
# MFI of CD 49b was significantly different (p=0.001) between C/C vs. C/T, C/C vs. T/T, and C/T vs. T/T.
Maximal aggregation
(% light transmission)

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<tr>
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<th>Heps Thrombin (U/mL) 40 20 10</th>
<th>Collagen (μg/mL) 40 20 10</th>
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<tr>
<td>C/C</td>
<td>6.0 ± 0.8</td>
<td>90.6 ± 4.7</td>
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<tr>
<td>C/T</td>
<td>2.8 ± 0.4</td>
<td>98.7 ± 6.3</td>
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<tr>
<td>T/T</td>
<td>3.0 ± 1.0</td>
<td>91.5 ± 3.5</td>
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<th>Thrombin (U/mL) 40 20 10</th>
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Results

Characteristics of the Study Population

Table 1 shows the characteristics of the study subjects. No significant differences were found in the prevalence of selected risk factors for arterial thrombosis, among patients and controls in the CHD and CVD case/control studies.

Prevalence of the 807 C/T Polymorphism in Normal Population and Patients with Thrombotic Diseases

The genotype and allelic frequencies of the 807 C/T polymorphism in the normal population and in the case/control studies are summarized in Table 2. The C allele was found in 65% of the 284 blood donors analyzed, whereas the T allele was present in 35% of these individuals. The prevalence of each 807 C/T genotype did not significantly differ in either CHD, CVD or in DVT patients with respect to their matched control groups, and were also similar to that found in the normal population. Finally, we performed prespecified subgroup analyses with stratification by age (≤60, >60), sex, type of coronary or cerebrovascular acute event, but we did not detect differences in the genotype or allele frequency associated with age, sex, or type of coronary or cerebrovascular acute event (data not shown).

Association of the 807 C/T Polymorphism with the Platelet Surface Expression of GPs

Flow cytometric analysis performed in platelets from blood donors demonstrated no major influence of the 807 C/T genotype in the platelet expression level of the GP IIIa and GP IV (Table 3). By contrast, we observed that the platelet surface content of the GP Ia differed significantly between carriers of each 807 C/T genotype. Subjects with T/T genotype presented the highest GP Ia values, followed by C/T carriers, and subjects with C/C genotype (Table 3). Concordant findings were obtained by measuring the platelet GP Ia content by hot binding assays using the anti-GP Ia monoclonal antibody 129-P116. Thus, in six individuals homozygous for the C allele we determined 2600 ± 846 GP Ia molecules/platelet, in comparison to 3967 ± 198 found in 4 C/T individuals, and 6622 ± 2167 detected in 2 T/T subjects.

Relationship of the 807 C/T Polymorphism and GP Ia Expression in other Cell Types

In order to elucidate the association of the 807 C/T genotype with the GP Ia expression in other blood cell types, we analyzed the expression of this molecule in monocytes and activated T-cells from blood donors with all distinct 807 C/T genotypes. Despite the weak expression of GP Ia on monocytes (significantly lower than in platelets), we observed that monocytes from C/C subjects express lower levels of GP Ia than monocytes of individuals carrying the T allele, although this difference did not reach statistical significance (Table 3). Our study showed that T cells activated with allogeneic EBV-LCL cells had detectable surface expression of the GP Ia after culture for at least 7 days, reaching a maximal level at day +20. Interestingly, we found that, as in platelets, the C/C carriers presented significant lower levels of GP Ia than C/T or T/T carriers in allogeneic EBV-LCL activated T-cells (CD49b MFI: 7.6 ± 2.1 vs 12.2 ± 0.7 respectively, p = 0.03) (Table 3).

Aggregability and Activation Profiles in Platelets with Different 807 C/T Genotype

Table 4 summarizes the aggregation response of washed platelets from blood donors with different 807 C/T genotype. Neither spontaneous platelet aggregation nor significant changes in the thrombin-induced aggregation response, were observed in association with the 807 C/T genotype. Moreover, although platelet responsiveness to type I collagen displayed considerable variability, we could not demonstrate significant differences between CC, CT or TT subjects in the maximal aggregation induced by graded concentrations of this agonist. Never-

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Table 5 Activation profile of platelets from blood donors with different 807 C/T genotype

Results are given as mean ± SD.

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theless, a delay in initiation of aggregation seems to be associated with 807 C/T genotype (Table 4). Thus, upon stimulation with 40 μg/ml of type I collagen, C/C platelets achieved 10% of aggregation following 119.2 ± 73.4 s, in comparison to the 72.5 ± 35.2 s needed by platelets from T allele carriers (C/T + T/T) to reach the same aggregation (p = 0.03).

We further compared the reactivity of platelets with different 807 C/T genotype, by analysis of the surface expression of CD62 and CD63 antigens, induced by several agonists. As shown in Table 5, either under static conditions or with continuous stirring in the presence of Lj-CP8, the spontaneous, thrombin or collagen-induced platelet activation showed no significant differences in relation to the 807 C/T genotype.

Discussion

We have analyzed the genotype and allelic frequencies of the recently described 807 C/T polymorphism (Phe224) of the collagen receptor GP Ia in 284 Spanish Caucasian blood donors. The 807 C/T polymorphism showed in our population a similar distribution than that described in 65 American individuals (10). The C allele was the most common with a gene frequency of 0.65, whereas the frequency of the T allele was 0.35. Moreover, we further confirmed the association of this polymorphism with the platelet GP Ia density in 159 blood donors. The presence of a T allele was statistically associated with high level of this receptor at platelet surface (p <0.0001). Since a neutral polymorphism cannot be responsible of such effect, the 807 C/T polymorphism should be a genetic marker of an unknown change able to influence the density of GP Ia in the platelet surface. To provide further insight to this issue, we have investigated the effect of this polymorphism in other blood cells expressing the GP Ia, such as monocytes and activated T lymphocytes (24-26). Interestingly, we found that the 807 C/T genotype could also be associated with the GP Ia density in activated T-cells, and probably in monocytes. These findings suggest that the yet unknown molecular change(s) primary responsible for the expression degree of the collagen receptor GP Ia, linked to the neutral 807 C/T polymorphism, could not be restricted to the megakaryocyte-specific regulatory elements of the promoter region of the GP Ia gene (27, 28).

The functional effects of different GP Ia density in cells expressing this protein, specially any putative change in their adhesive properties, deserves further investigation. Noteworthy, the number of GP Ia on platelet surface has been previously shown to influence the platelet adhesion (4-10). Therefore, and considering the importance of the platelet adhesion in all subsequent steps of thrombus formation, a high density of GP Ia might alter the platelet activation and aggregation. However, from our "in vitro" study, the only apparent effect of the 807 C/T polymorphism was a variation in the time for starting the aggregation process upon stimulation with collagen. Very recently, adhesion data from perfusion experiments using platelets with different 807 C/T genotype seem to be in agreement with our results (29). These authors found a delay in the adhesion of C/C platelets to collagen surface compared to T/T platelets. However, after 3 min of perfusion, the surface coverage is similar between T/T and C/C platelets. Thus, subjects bearing few GP Ia receptors (807 C/C genotype) apparently need more time to bind enough collagen to initiate aggregation. However, once the initial signal was achieved, the maximal aggregation was similar in all subjects independently of the 807 C/T genotype. This apparent delay in the collagen-induced aggregation response was not extensive to other agonists such as ADP or thrombin, thus supporting its relationship with the reduced level of the collagen receptor GP Ia. The platelet activation profiles induced by collagen under static or stirring conditions confirmed these results, as we did not observe significant changes between platelets with different GP Ia density. Indeed, subjects with very low number of receptors (500 per platelet) displayed analogous externalization of the CD62 and CD63 activation markers in response to collagen, than subjects with a 10-fold platelet content of GP Ia (5000 per platelet). These "in vitro" findings suggest a minor role of GP Ia in the collagen-induced degranulation and final aggregation of platelets, which might rather be mediated by some other collagen receptors.

Nowadays, there is considerable interest on the role of polymorphisms of plasma proteins (30-37), endothelial cell surface glycoproteins (38-41), and platelet receptors (42, 43), as putative risk factors for thrombosis and/or atherothrombosis. To our knowledge, this is the first study addressing whether the 807 C/T polymorphism is an independent genetic risk factor for arterial and/or venous thrombosis. We performed three case-control studies for CHD, CVD and DVT involving 302 patients designed to achieve similar prevalence of several risk factors between patients and controls (Table 2). Our studies found no significant differences in the prevalence of any 807 C/T genotype or allele between patients and controls for any thrombotic disorder (CHD, CVD and DVT), and their frequencies were similar to that found in healthy population. Thus, this polymorphism seems to play no major role in the development of arterial or venous thrombosis in the Mediterranean Caucasian population.

In summary, this study provides new data showing that the 807 C/T polymorphism associates with different expression level of GP Ia in platelets and other cells. Our "in vitro" studies show no major functional differences between platelets with distinct 807 C/T genotype, thus supporting the idea that although GP Ia/Iib is involved in primary adhesion to the collagen matrix, only minimal collagen-GP Ia/Iib interactions might be needed to initiate the subsequent steps that result in platelet aggregation (44). Finally, our case-control studies provide no evidence to consider the 807 C/T polymorphism as a major genetic risk factor for thrombotic disease.

Acknowledgements

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