In vitro effect of anti-β_2 glycoprotein I antibodies on P-selectin expression: a marker of platelet activation*

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SUMMARY

Objective. Antiphospholipid antibodies (aPL) associated with thrombembolic events and/or pregnancy morbidity characterize the so-called antiphospholipid syndrome (APS). Beta2glycoprotein I (β_2GPI) represents the major target antigen for aPL, but the pathogenic role of anti-β_2GPI antibodies (aβ_2GPI) is still unclear. Some authors assume they play a role in activating platelets. The effects of aβ_2GPI antibodies on platelet P-selectin expression were evaluated in this study.

Methods. Aβ_2GPI antibodies in the plasma of a pregnant APS patient were isolated by affinity chromatography during two different stages (catastrophic and quiescent) of the disease. Gel filtered platelets (100,000/µl) from healthy volunteers were incubated with β_2-GPI (20 µg/ml) and with different concentrations (5, 25 e 50 µg/ml) of aβ_2GPI antibodies. P-selectin surface expression on platelets was assessed by flow cytometry using a specific fluorescent antibody directed against P-selectin.

Results. Aβ_2GPI antibodies induced platelet activation only in the presence of thrombin receptor activator for peptide 6 (TRAP-6), a platelet agonist, at a subthreshold concentration. Aβ_2GPI antibody enhancement on platelet surface P-selectin expression was stronger in the catastrophic than in the quiescent phase of the disease (47% versus 15%).

Conclusions. TRAP-6 dependent platelet activation by aβ_2GPI antibodies is consistent with the "two hit" pathogenetic hypothesis for thrombosis. Aβ_2GPI antibodies induce higher platelet P-selectin expression during the active rather than in the acute phases.

Key words: *anti-β_2Glicoprotein I antibodies, P-selectin, platelet activation, antiphospholipid syndrome.*

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INTRODUCTION

Antiphospholipid antibody syndrome (APS) is an autoimmune disease characterized by thrombotic events (arterial, venous, or of the microcirculation) and/or of obstetric complications (repeated abortion, loss of fetus ≥10 sg, or preterm birth) associated with the presence of antiphospholipid antibodies in the blood (aPL). Principle aPL are the adnticariolipin antibodies (aCL), anti-beta2glycoprotein I (antiβ_2-GPI) and lupus anticoagulants (LA) (1). The antigen targets of the aPL are complexes costituted by phospholipids and plasma proteins such as prothrombin, anexin V and above all beta2-glycoprotein I (β_2-GPI) (2, 3). Beta2-glycoprotein I is composed of a single proteic chain of 50 kDa subdivided into five domains, they are found in plasma in concentrations of approximately 4 µM and inhibit platelet aggregation and relase of inflammatory mediators (4, 5). The pathogenetic significant of the anti-β_2GPI (aβ_2GPI) antibodies is still not clear. It is thought that they are capable of activating platelets (6-10) which release a series of inflammatory mediators such as P-selectin (P-sel). This mediates bonding of leukocytes to endothelial cells and activated platelets (11, 12) the matrix metalloproteinase type 2 (MMP2) (13, 14) and the CD40 ligand (CD40L) (15). Accelerated atherosclerosis has been described

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in patients with APS; therefore, the effect of the aβ_2GPI antibodies on platelets could play a role in accelerating the atherogenesis. In fact, it has been reported (16) that activated platelets could trigger inflammation of the vascular endothelium with subsequent artherosclerotic lesions and/or atherothrombosis.

aPL seem to exercise a thrombophilic action which also involves the endothelial cells. In fact, *in vitro* and *in vivo* studies (17, 18) have shown that they activate the endothelium and promote the expression of adhesion molecules.

Traditional antithrombotic treatment is not always sufficient to deal with the most serious complications of patients with APS, whether they be of a vascular or obstetric nature. In particular, the biggest problems from a therapeutic point of view are to be found in the management of patients with catastrophic APS. This is a clinical syndrome characterized by multiple thrombotic involvement, and mainly thrombosis of the microcirculation. Mortality rates are 50% in spite of administration of adjunctive therapies to anticoagulatnt treatment, such as plasmapheresis, immunosuppressive drugs and bolus of intravenous immunoglobulin.

In this study, we evaluated the effect of aβ_2GPI antibodies on activation of platelets by measuring platelet P-selectin expression in order to examine the mechanisms by which aβ_2GPI antibodies are involved in the thrombotic complications which characterize APS.

MATERIALS AND METHODS

Purification of β_2-GPI antigen

β_2-GPI was purified by human group A-RH+ plasma. Plasmatic protein precipitation was performed using perchloric acid ($HClO_4$) at 65%. After buffering to pH 8.0 with a saturated sodium carbonate solution (Na_2CO_3) and dialysis with a sodium chloride buffer (NaCl) 0.03 M, a first ionic exchange chromatograph was performed using a GradiFrac (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), making use of the bonding of β_2-GPI to heparin. The protein was eluted with NaCl 0.35 M and underwent dialysis against NaCl 0.15 M. A second chromatograph was performed with a washing solution of NaCl 0.15 M and an elution solution of NaCl 0.35 M. The resultant sample underwent dialysis against an acetate buffer 0.05 M to pH 4.8, and was passed through a carboxymethylcellulose (CM-cellulose) column. After an hour of incubation, fractions with highest optical density at 280 nm were collected against Tris buffer at pH 7.4. Quality of antigen was evaluated on a polyacrylamide electrophoresis gel in the presence of sodium dodecyl sulfate (SDS-PAGE). Specificity of the antigen bond with aβ_2GPI antibodies was evaluated with a home-made ELISA assay following the indications of the European Forum on Antiphospholipid Antibodies (19). We tested 50 sera from patients positive for aβ_2GPI antibodies and 50 sera from patients negative for aβ_2GPI antibodies. The cut-off value had been determined previously using sera of 100 healthy subjects and calculating the 95th percentile as cut off for the low levels (1.62 U) and 99th percentiile as cut off for medium-high levels (2.65 U).

Estraction of aβ_2GPI antibodies

aβ_2GPI antibodies were extracted from the plasma of a 35-year old patient with APS in quiescent phase of the disease and from plasma of the same patient in catastrophic phase of the disease. APS was diagnosed in primary form in this patient at the age of 21 years on the basis of relapsed venous thrombosis involving the lower limbs in association with blood tests confirming aβ_2GPI and high IgG aCL antibodies (1085 U and 1147 GPL, respectively) and positivity for lupus anticoagulant. The catastrophic phase of the disease developed at the moment of giving birth and was characterized by thrombosis of the microcirculation at the extremities of the upper and lower limbs, and of signs of hepatic and renal failure. The patient presented hyperpyrexia, piastrinopenia and signs of coagulation activation such as antithrombin III consumption. On presentation of the catastrophic phase the antiphospholipidic patterns was still made up of triple positivity

and aβ_2GPI and aCL antibody levels which, although still high, were lower than those seen in the quiescent phase (139 U and 221 GPL, respectively) due to the removal of the aPL from the circulation by the apheretic treatment given during pregnancy. The plasma was passed through an affinity column coated with β_2-GPI antibodies (Hi-Trap NHS-activated, GE Healthcare). After an hour of incubation, the aβ_2GPI antibodies were eluted with a glycine buffer at pH 2.8 and, finally, the eluate containing the aβ_2GPI antibodies was buffered with a Tris 1 M solution at pH 8. Antigen specificity of the purified antibodies was confirmed by a home-made ELISA assay. The efficacy of the bonding of the purified antibodies with the β_2GPI was evaluated using 8 different antibody concentrations and it was seen that the best bonding was obtained with a concentration of between 5 and 50 μg/mL.

Study of platelet P-selectin expression by flow cytometry

To study platelet P-selectin expression, platelets from a healthy donor were used as follows: gel filtered ($100x10^9$/L), aβ_2GPI antibodies at different concentrations (5, 25 and 50 μg/mL) and, since a significant activation was not obtained in the presence of the only native antigen which adhered to the platelet membrane, it was decided to add 20 μg/mL of β_2-GPI antigen. Samples were incubated for 30 min at 37°C. Then, 5 μL of sample, 5 μL of direct antibody against platelet-specific antigen (anti-CD41) marked with phycoerythrin (PE) and 5 μL of anti-P-selectin antibody marked with isothiocyanate of fluorescein (FITC) were placed in test tubes containing phosphate buffer saline (PBS). After 30 min of incubation in the dark at room temperature, the samples were fixed by the addition of 1% paraphormaldeide (PFA) 1% and analyzed with a Coulter EPICS-XL flow cytometer (Beckman, GMI Inc., USA) with a 488 nm argon laser. The resultant fluorescence from the two fluorochromes were read by a 575 and 525 nm filter laser band pass. Platelets were identifed on the basis of morphological characteristics and positivity for CD41. Platelet P-selectin expression was expressed as percentage of positive platelets. We also evaluated whether aβ_2GPI antibodies were able to potentiate platelet activation induced by a weak stimulus. Preliminary experiments were performed in order to identify the concentrations of the thrombin receptor activator for peptide 6 (TRAP-6), an activator of the receptor activated by protease 1 (PAR1). The results showed that at a concentration of 3 μM, TRAP-6 induced P-selectin expression of between 30 and 50% of the maximum value. This concentration was, therefore, used to stimulate the platelets. All experiments were repeated three times, always using double testing procedures.

RESULTS

Results obtained are shown in Figures 1 and 2 with mean and standard deviation of percentages of platelets positive for P-selectin. The figures show different values because of the different platelet reactivity of the donors. In fact, the donor platelets used for the experiments with the aβ_2GPI antibodies from the patient in catastrophic phase of the disease were less reactive. However, this difference did not influence the results or conclusions of the study because all samples were tested on platelets from the same donor. Results showed that aβ_2GPI antibodies alone have no effect on platelet P-selectin expression. In fact, the percentage of platelets positive to P-selectin did not show any variation in the presence of aβ_2GPI antibodies at three different concentrations (5, 25, 50 μg/mL).

In contrast, as shown in Figure 1, in subthreshold levels of the TRAP-6 agonist (3 μM), aβ_2GPI antibodies isolated from the plasma in the quiescent phase of the disease induced a 15% increase in platelet P-selectin expression with respect to the sample incubated in the presence of a single vehicle (Tris/BSA 1%) when used at a concentration of 25 μg/mL (Figure 1). Experiments were also performed which showed that control antibodies (normal human immunoglobulin) had no effect on platelet P-selectin expression induced by

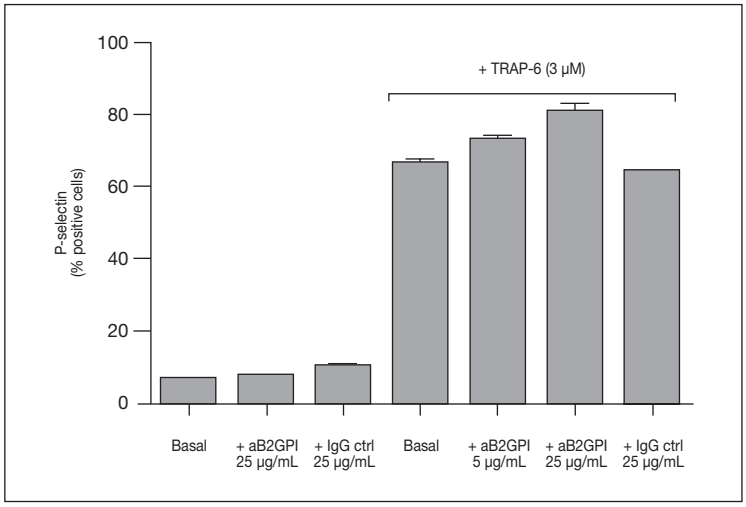

Figure 1 - Effect of anti-beta2glycoprotein I antibodies (antiβ2-GPI), isolated from plasma from a patient with APS in quiescent phase of the disease, on platelet activation, expressed as percentage of platelets postive for P-selectin (P-sel). aβ2GPI amtibodies alone had no effect on platelet P-selectin expression. In fact, the percentage of platelets positive for P-selectin showed no variation in the presence of aβ2GPI. After addition of thrombin receptor activator (TRAP-6), aβ2GPI antibodies induced a 15% increase in platelet P-selectin expression with respect to the sample incubated in the presence of a single vehicle (Basal) and of the sample with control antibodies (IgG ctrl).

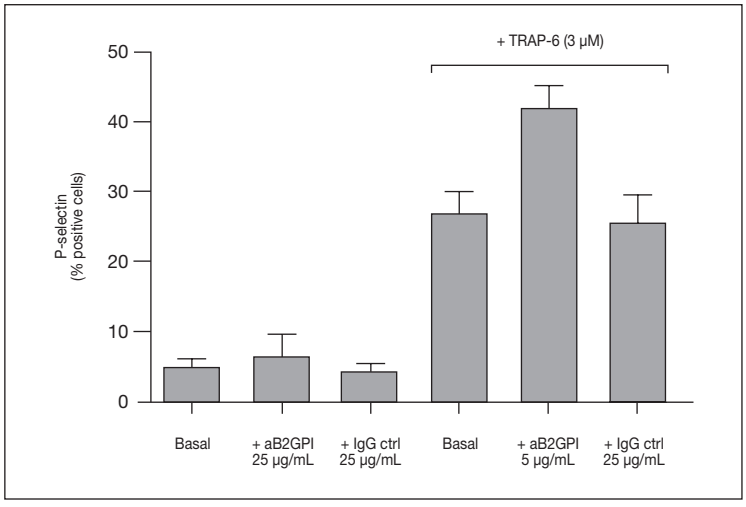

Figure 2 - Effects of anti-beta2glycoprotein I antibodies (antiβ2-GPI), isolated from plasma of a patient with APS in catastrophic phase, on platelet activation expressed as percentage of platelets positive for P-selectin (P-sel). aβ2GPI alone had no effect on platelet P-selectin expression. In fact, there was no variation in the percentage of platelets positive for P-selectin in the presence of aβ2GPI. After the addition of thrombin receptor activator (TRAP-6), aβ2GPI antibodies induced a 47.7% increase in platelet P-selectin expression with respect to the sample incubated in the presence of single vehicle (Basal) and the sample with control antibodies (IgG ctrl).

TRAP-6 at any of the concentrations tested (Figure 1). The same experiments were repeated incubating the platelets with aβ_2GPI antibodies isolated from the plasma during the catastrophic phase of APS (Figure 2). The results showed that also these antibodies alone had no effect on platelet activation. On the other hand, always in the presence of subthreshold doses of TRAP-6, already at a concentration of 5 μg/mL, platelet P-selectin expression increased by 47.7% (Figure 2). Antibodies purified during the catastrophic phase of the disease, therefore, strengthened platelet activation at lower concentrations than those found in purified antibodies from subjects with APS in the quiescent phase of the disease.

DISCUSSION

Our results show that the trigger for platelet activation requires the addition of the TRAP-6 antagonist and that only after this were aβ_2GPI antibodies strengthened. These results afree with the "two hit theory" for triggering thrombotic events in APS, according to which only in the presence of triggers (infections, stress, drugs, etc.) can the aPL activate thrombosis (20-22). It is interesting to observe that in order for aβ_2GPI antibodies to activate platelets it is necessary to add extracted β_2GPI antigen to the native antigen adherent to the platelets. This could support the hypothesis of some authors (6, 7) who report that platelet activation is induced by the union of aβ_2GPI antibodies to the antigen in dimeric form which would then bond to the receptors of the Ibα glycoprotein platelet membrane and apolipoprotein E receptor 2' modifying hemostatis in favor of thrombosis.

We have also observed the the aβ_2GPI antibodies present in the catastrophic phase of APS with respect to the quiescent phase seem to lead to greater platelet activation, even when used at lower concentrations. This is of great clinical interest because it could explain the serious thrombophilia associated with the catastrophic phase of APS. The data emerging from this study agree with the pathogenetic hypothesis that attri-

butes an active role to aPL in the pathogenesis of thrombosis through platelet activation (9, 10). However, this needs further verification on larger cohorts of subjects both in acute phase and in quiscent phase of APS. Finally, in order to confirm this hypothesis, it could be useful to also evaluate other markers of platelet activation, such as expression of MMP2 and CD40L, and the production of platelet-derived microparticles.

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