

Role of β_2 -glycoprotein I and anti-phospholipid antibodies in activation of protein C in vitro

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Abstract

Aims—To investigate the effect of β_2 -glycoprotein I (β_2 GPI) on the thrombin/thrombomodulin dependent activation of protein C; and to determine whether β_2 GPI dependent anticardiolipin antibodies have any effect.

Methods—Protein C was activated by thrombin in the presence of thrombomodulin and phospholipid vesicles in an in vitro system. The effect of adding purified β_2 GPI to this system was observed. Affinity purified anticardiolipin antibodies and total IgG from patients with anticardiolipin antibodies and the lupus anticoagulant were studied for their effects on protein C activation in the presence and absence of β_2 GPI.

Results— β_2 -Glycoprotein I had no effect on the activity of preformed activated protein C. When the phospholipid vesicles were incubated with β_2 GPI before the addition of protein C, the activation of protein C was inhibited in a dose dependent manner. With phosphatidylserine:phosphatidylcholine vesicles at a concentration of 1 μ M:2 μ M, β_2 GPI began to inhibit the reaction at a concentration of 15 nM, and at 4 μ M (the normal plasma concentration) the activation of protein C was reduced to 40%. Anticardiolipin antibodies had no demonstrable effect.

Conclusions— β_2 -Glycoprotein I inhibits protein C activation in an in vitro system. Its physiological role is unknown but it has potential procoagulant as well as anticoagulant properties. An effect of antiphospholipid antibodies on protein C activation, which might explain their association with thrombosis, could not be shown.

(J Clin Pathol 1993;46:908-911)

Antibodies to phospholipid (aPL) are associated with venous and arterial thrombosis¹⁻⁴ and recurrent fetal loss.^{5,6} Fetal loss may be due to thrombosis of placental vessels.⁷ These antibodies are identified in two different ways, either as immunoglobulins reacting with cardiolipin (aCL) or other anionic phospholipids (usually detected in solid phase enzyme linked immunosorbent assay), or by their ability to prolong phospholipid dependent coagulation tests, the so-called lupus anticoagulants. There have been many

hypotheses suggesting mechanisms of action whereby these antibodies might cause or be associated with thrombosis. One of the possibilities is interference with the activation of protein C by the thrombin/thrombomodulin (IIa/TM) complex,^{8,9} a reaction that is enhanced by phospholipids.¹⁰ Activated protein C inactivates factors Va and VIIIa and stimulates fibrinolysis,^{11,12} and deficiency is a risk factor for thrombosis.¹³

Recently it has been shown that purified aPL only bind to anionic phospholipids in the presence of a serum cofactor,¹⁴⁻¹⁶ identified as β_2 -glycoprotein I (β_2 GPI).¹⁴ A protein that forms a complex with activated protein C via its active site has been described¹⁷ and this seems to be β_2 GPI.¹⁸ We therefore decided to investigate whether β_2 GPI affected either the rate of protein C activation by the IIa/TM complex or the activity of activated protein C, and to determine the effect of aPL on protein C activation both in the presence and the absence of β_2 GPI.

Methods

Plasma was obtained from five female and one male patient who all had aCL and lupus anticoagulants. Their details have already been published.¹⁹

Cardiolipin, phosphatidylserine, phosphatidylcholine, bovine serum (B-2771), bovine albumin (7030), human thrombin and hirudin were obtained from Sigma Chemical Company Ltd, Poole, Dorset; rabbit lung thrombomodulin from American Diagnostica Inc., Greenwich, Connecticut, USA; human protein C from CRTS, Lille, France; protein G, S sepharose fast flow, heparin sepharose, and sephacryl S200 HR from Pharmacia, Milton Keynes, Bucks; Protein C Substrate (2AcOH·H-D-Lys(Cbo)-Pro-Arg-pNA) and the reagents used in the diluted Russell's viper venom test (DRVVT) were from Unicorn Diagnostics, London.

For cardiolipin affinity chromatography, the method of McNeil *et al*⁴ was used, though with step rather than gradient elution. The cardiolipin affinity column was equilibrated with 0.02 M TRIS (pH 7.2). Plasma was diluted 1 in 5 with this buffer and applied to the column. After washing, bound protein was eluted with 0.02 M TRIS/0.75 M NaCl (pH 7.2).

For affinity purification of anticardiolipin antibodies 10 ml plasma from the six patients were chromatographed on the cardiolipin affinity column. The protein eluted was dia-

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Accepted for publication
5 May 1993

lysed against 0.02 M TRIS (pH 7.2) and applied on to a protein G column. IgG was eluted off the protein G with 0.1 M glycine/HCl (pH 7.2). The antibodies were concentrated in 0.02 M TRIS/0.15 M NaCl (pH 7.4) (TBS) using an Amicon Mini-Ultrafiltration Cell with a PM30 membrane to 0.15 g/l (1 μ M) assuming an extinction coefficient of 13.6 at 280 nm for a 1% solution.

β_2 -glycoprotein I (β_2 GPI), 0.5 g/l (10 μ M) in TBS was purified by a previously described method,²⁰ modified from that of McNeil *et al.*¹⁴

Total IgG was prepared from the plasma of the six patients using the protein G column and concentrated to 3 g/l (20 μ M).

The antiphospholipid enzyme linked immunosorbent assay (ELISA) was based on that of Gharavi *et al.*,²¹ using 10% (v/v) bovine serum as the blocking agent and sample diluent. Blanks obtained from uncoated wells on the same plate were subtracted to account for non-specific binding. The reference serum was calibrated at 105 GPL against a standard prepared at an international workshop²² (GPL are arbitrary units derived from the activity of an affinity purified serum).

In our modified DRVVT 0.1 ml affinity purified aCL (AP-aCL), or total IgG, or buffer, was mixed with 0.1 ml normal plasma and 0.1 ml of dilute phospholipid reagent (or freeze-thawed platelets); 0.1 ml Russell's viper venom was then added and after incubation for 30 seconds clotting was initiated by adding 0.1 ml of 25 mM CaCl₂.

Stock solutions of phospholipids in organic solvents were mixed to give the desired molar

composition. The mixture was dried in nitrogen and the residue dispersed in TBS to give the following concentrations: phosphatidylserine:phosphatidylcholine 25 μ M:50 μ M; cardiolipin:phosphatidylcholine 50 μ M:200 μ M; cardiolipin alone 200 μ M. The emulsion was sonicated for 30 seconds every minute for 40 minutes under nitrogen at 27°C using an MSE Soniprep 1500 at an amplitude of 6 μ M. The resulting unilamellar vesicles were kept at room temperature before use.

Activation of protein C was carried out in TBS (pH 7.4) containing 0.5 g/l bovine albumin: 100 μ l CaCl₂ (25 mM), 10 μ l phospholipid vesicles, and 10 μ l TM (1.25 U/ml) were incubated for 15 minutes. TBS (110 μ l) with or without β_2 GPI or IgG was then added and after 15 minutes 10 μ l IIa (25 U/ml) and 10 μ l protein C (25 U/ml) were added to start the reaction. Final concentrations were CaCl 10 mM, TM 0.05 U/ml, IIa 1 U/ml, protein C 1 U/ml; and for the different phospholipid vesicles, phosphatidylserine:phosphatidylcholine 1 μ M:2 μ M, cardiolipin:phosphatidylcholine 2 μ M:8 μ M, cardiolipin alone 8 μ M. After 15 minutes 80 μ l samples were taken in duplicate and added to 20 μ l hirudin (10 U/ml) in a microtitre plate to inactivate the thrombin. Protein C (50 μ l) substrate (1.8 mM) were added and after incubating for 15 minutes at 37°C cleavage of substrate by preformed activated protein C was stopped by adding 50 μ l of 1 M citric acid. The activated protein C content was then proportional to the absorbance measured at 405 nm. The amount of activated protein C formed in an experiment was expressed as a percentage of that obtained in a control, run concurrently, under these standard conditions with no β_2 GPI or immunoglobulin added. Each of the phospholipid vesicles at these concentrations enhanced the reaction about five-fold—that is, in the absence of phospholipid vesicles protein C activation as defined above was about 20%.

Results

When β_2 GPI was added at the end of the reaction, at a final concentration of 4 μ M, it had no effect on the activity of preformed activated protein C (data not shown).

When the phospholipid vesicles were incubated with β_2 GPI before the addition of protein C, the activation of protein C was inhibited in a dose dependent manner. This inhibition was seen with all three phospholipid vesicles (fig 1). All further experiments were performed with the phosphatidylserine:phosphatidylcholine vesicles. With these vesicles at a concentration of 1 μ M:2 μ M, β_2 GPI began to inhibit the reaction at a concentration of 15 nM, and at 4 μ M the activation of protein C was reduced to 40%. At a β_2 GPI concentration of 0.1 μ M the inhibition could not be completely overcome by increasing the phospholipid concentration (fig 2).

The activities of the IgG preparations in the aCL ELISA and in our modified DRVVT

Figure 1 Protein C activation (as a percentage of that without β_2 GPI) v β_2 GPI concentration (log scale) for the three phospholipid vesicles, phosphatidylserine:phosphatidylcholine (●, —), cardiolipin (+, ---) and cardiolipin:phosphatidylcholine (Δ , ---). Results are the mean of five experiments.

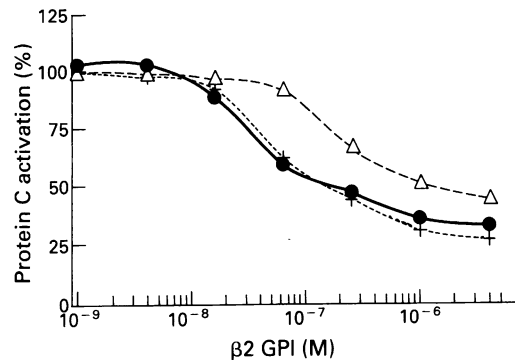


Figure 2 Protein C activation (as a percentage of that with 1 μ M phosphatidylserine without β_2 GPI) v phospholipid concentration (log scale), without β_2 GPI (●) and with 0.1 μ M β_2 GPI (○). Results are the mean of two experiments.

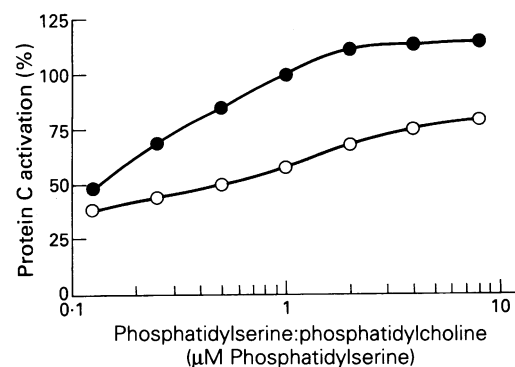


Table 1 ACL levels and DRVVT ratios with dilute phospholipid (PL) and freeze thawed platelets (platelets) for AP-aCL and total IgG purified from six patients

Case No	aCL (GPL)		DRVVT AP-aCL		DRVVT total IgG	
	AP-aCL	Total IgG	PL	Platelets	PL	Platelets
1	139	89	1.42	0.92	1.43	0.91
2	135	126	1.53	1.00	1.14	0.99
3	33	18	1.18	1.06	2.01	1.25
4	110	72	1.34	0.99	1.27	1.07
5	38	19	1.06	0.93	1.11	0.99
6	23	16	0.94	0.95	1.04	0.89

Results of the mean of the two experiments.

Table 2 Protein C activation in the presence of aPL with and without β_2 GPI, expressed as percentage of that obtained in absence of immunoglobulin and β_2 GPI

Case No	AP-aCL		Total IgG	
	Alone	+ β_2 GPI	Alone	+ β_2 GPI
1	107	70	104	72
2	96	68	92	63
3	114	84	90	66
4	101	78	103	73
5	113	84	102	71
6	95	83	90	56
Normal IgG mean (SD)	97 (8.6)	68 (8.2)	95 (7.8)	64 (7.8)

Results for the patient IgG are the mean of two experiments, for normal IgG at the appropriate concentration the mean (SD) of five experiments.

are shown in table 1. The effect of each on protein C activation was studied. In each case 100 μ l of the immunoglobulin was used to give a final concentration of 0.4 μ M for the AP-aCL and 8 μ M for the total IgG. Experiments were done without β_2 GPI and with β_2 GPI at a final concentration of 0.04 μ M, which in the absence of immunoglobulin gave a protein C activation of 74% (n = 5, SD = 6.8%) and was at a point on the steep part of the inhibition curve (fig 1). Neither the AP-aCL nor the total IgG preparations had a demonstrable effect (table 2). In the absence of β_2 GPI the mean (SD) protein C activation for the six AP-aCL and the six total IgG preparations was 104% (8.3%) and 97% (6.8%) and in the presence of β_2 GPI 78% (7.2%) and 67% (6.6%), respectively. These results do not differ significantly from those without any immunoglobulin, nor from those with normal IgG at the appropriate concentration (t test; all p > 0.05).

Discussion

β_2 GPI binds to phospholipids,^{23,24} platelets,²³ and perhaps activated protein C,^{17,18} and has been found to have several effects on the coagulation system. It inhibits the contact activation of the intrinsic pathway of coagulation²⁵⁻²⁸ and it interferes with the assembly of the prothrombinase complex on platelet membranes and phospholipid vesicles.²⁹ We could not detect any inhibition of preformed activated protein C at physiological concentrations of β_2 GPI. It might, however, be expected to inhibit the IIa/TM/phospholipid dependent activation of protein C, very much like it inhibits prothrombinase, simply by occupying the phospholipid surface. We initially used the three different phospholipid

vesicles in case the phospholipid phase had any effect. Pure cardiolipin vesicles in the bilayer phase undergo transition to the hexagonal phase when calcium is added; the cardiolipin:phosphatidylcholine mixture is stabilised and remains in the bilayer phase when calcium is added as do the more physiological phosphatidylserine:phosphatidylcholine vesicles. Whichever vesicles were used, β_2 GPI resulted in decreased protein C activation at low concentrations (the normal plasma concentration is 4 μ M). These results contrast with those of Oosting *et al.*,³⁰ who found no effect of β_2 GPI on endothelial cell mediated protein C activation, using a more physiological in vitro system. Clearly, our results do not mean that β_2 GPI inhibits protein C activation in vivo, but they show that β_2 GPI has the potential to interfere with anticoagulant as well as procoagulant pathways. On the basis of their in vitro data, workers have naturally speculated that β_2 GPI may act as a physiological anticoagulant,^{25,29} but this may not be so. Indeed, the plasma concentration of β_2 GPI depends on genotype; homozygotes for the normal gene (BgN, gene frequency 0.937) comprise 88% of the population and have concentrations of 150–300 mg/l. Homozygotes for the BgD gene (gene frequency 0.063) have concentrations of 0–50 mg/l and would be expected to comprise 0.4% of the population, and heterozygotes have intermediate values, 50–150 mg/l, and comprise 12% of the population. Despite this there are no reports of thrombosis associated with low β_2 GPI concentrations and one report which suggests such an association is unlikely.³¹

In 1986 Freyssinet *et al.*⁸ isolated a lupus anticoagulant fraction from a patient's plasma by gel filtration. They studied the effect of this IgM containing fraction on the IIa/TM dependent activation of protein C, using human derived purified proteins. They found that the anticoagulant could neutralise the enhancement of protein C activation seen with phospholipids. In 1988 Cariou *et al.*⁹ purified IgG from eight patients with the lupus anticoagulant on protein A sepharose. They studied the activation of protein C by thrombin in the presence of human endothelial cells or rabbit thrombomodulin. In both systems the IgG with lupus anticoagulant activity inhibited activation of protein C. In contrast, Oosting *et al.*³⁰ could not detect an effect for aPL on endothelial cell mediated protein C activation in the presence or absence of β_2 GPI. They studied 46 sera from patients with systemic lupus erythematosus, 19 of whom had aCL or lupus anticoagulant activity, and purified total IgG on protein G sepharose from 12 of them, six of whom had aCL or lupus anticoagulant activity. We, too, could not show an effect on protein C activation with either AP-aCL or total IgG from our patients. Some of these disparate results may be due to the heterogeneity of aPL, an understanding of which is beginning to emerge. Bevers, Galli, and colleagues have proposed that the term lupus anticoagulant

is used to describe those aPL which prolong coagulation tests because they recognise a prothrombin-Ca²⁺-phospholipid complex³² and that aCL which recognise a β_2 GPI-phospholipid complex are divided into aCL-type A which prolong phospholipid dependent coagulation reactions and aCL-type B which have no anticoagulant activity.³³ It seems likely that some of these patients will have antibodies against other protein-phospholipid complexes, such as factor X-Ca²⁺-phospholipid (which would be a lupus anticoagulant) and protein C-Ca²⁺-phospholipid; indeed antibodies against prothrombin, factor X, and protein C could be cross-reactive. Our six patients all had aCL and lupus anticoagulant activity. Their total IgG could contain any of the antibody types mentioned. We affinity purified aCL in the absence of calcium, so antibodies which recognise a protein-Ca²⁺-phospholipid complex could not copurify. The resulting antibodies have already been characterised. They recognise phospholipid bound β_2 GPI and in cases 1-4 the lupus anticoagulant activity is probably due to aCL-type A.^{19,20} This could not be shown in cases 5 and 6, and in these patients it may have been that the lupus anticoagulant activity was not due to antibodies against β_2 GPI. Unfortunately the total IgG from case 5 had extremely weak lupus anticoagulant activity and that from case 6 none at all (the antibodies may have been inactivated during purification or they may have been IgM). We have therefore not been able to show an effect with aPL which recognises a β_2 GPI-phospholipid complex, but cannot exclude the possibility that antibodies to other protein-phospholipid complexes affect protein C activation.

Finally, an inability of aPL to impair protein C activation does not mean that they do not interfere with the activated protein C/protein S mediated inactivation of factors Va and VIIIa, as has been suggested.³⁴⁻³⁶

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