Anti-tissue factor pathway inhibitor activity in subjects with antiphospholipid syndrome is associated with increased thrombin generation

Murray Adams  
Liza Breckler  
Paul Stevens  
Jim Thom  
Ross Baker  
Robert Oostryck

Background and Objectives. Immunoglobulin G (IgG) fractions from subjects with antiphospholipid syndrome (aPS) have previously been demonstrated to have inhibitory activity against tissue factor pathway inhibitor (TFPI). This may contribute to the development of a prothrombotic state by impaired regulation of the tissue factor (TF) pathway. This study investigated the effect that IgG fractions from aPS subjects containing anti-TFPI activity have on in vitro TF-induced thrombin generation.

Design and Methods. TFPI and anti-TFPI activities were determined in normal controls (n=29) and aPS subjects (n=57). TFPI activity was determined using an amidolytic assay based on the generation of factor Xa. Anti-TFPI activity was determined after incubating IgG isolated from a control or subject plasma with pooled normal plasma, using the TFPI activity assay. The influence of IgG fractions from controls (n=10) and subjects (n=23) on TF-induced in vitro thrombin generation was determined using a chromogenic assay of thrombin activity.

Results. TFPI activity in controls (1.13±0.25 U/mL) was significantly lower than in subjects (1.30±0.42 U/mL) (p<0.05). Anti-TFPI activity was significantly higher in subjects than controls (p = 0.0001). TF-induced thrombin generation was positively associated with anti-TFPI activity (p = 0.356; p > 0.05), with increased levels of each demonstrated in 5 subjects.

Interpretations and Conclusions. Anti-TFPI activity was confirmed in 65% of aPS subjects. IgG fractions demonstrated a variable ability to interfere with TFPI function and TF-induced thrombin generation. Cross-reacting antiphospholipid antibodies and/or other entities may interfere with TFPI function, resulting in a net increase in thrombin generation and an increased thrombotic risk.

Key words: antiphospholipid syndrome, TFPI, thrombosis, anti-TFPI activity.

The antiphospholipid syndrome (aPS) is a heterogeneous disorder characterized by circulating antiphospholipid antibodies (aPL), with venous or arterial thrombosis, recurrent miscarriages and thrombocytopenia.1 APL target phospholipid bound proteins, including β-2-glycoprotein-I (β2GPI) and prothrombin, with varying specificity.2 It has been proposed that aPL contribute directly to the clinical manifestations of aPS,2 though no single mechanism has been clearly identified. On the other hand, the heterogeneity of clinical manifestations of aPS probably reflects multiple contributing factors that lead to the development of hypercoagulability. These include, but are not limited to, interference with platelet function,2 inhibition of the protein C anticoagulant system2,4 and inhibition of fibrinolysis.2

Changes to the tissue factor (TF) pathway of coagulation have more recently been implicated in the development of hypercoagulability in aPS.5 Indeed, inhibitory activity (in IgG fractions) against tissue factor pathway inhibitor (TFPI)6 and autoantibodies (in plasma) to TFPI7,8 have been identified in aPS subjects. Whether these are actually the same entity remains to be elucidated, but it would seem feasible that anti-TFPI activity is due to the contribution of anti-TFPI antibodies and possibly other cross-reacting aPL. An impaired ability of TFPI to regulate the TF pathway, for example by an anti-TFPI antibody or another interfering factor, may contribute significantly to the development of a prothrombotic state. The aim of this study was, therefore, to investigate how in vitro TF-induced thrombin generation was affected by IgG fractions from aPS subjects containing anti-TFPI activity.
Design and Methods

Ethics
The study was approved by the Human Research Ethics Committee of Curtin University (Approval Number: HR 238/2001) and the Ethics Committee of Royal Perth Hospital (Reference: EC 2002/013).

Patients and controls
Venous blood was collected from 29 normal controls (M=18; F=11; mean age: 24.6±8.6 years) and 57 aPS subjects (M=28; F=29; mean age: 50.2±20.2 years). Subjects presented with a history of aPS-related clinical features or demonstrated aPS laboratory markers. Nine parts whole blood was collected into 1 part 0.109 mol/L tri-sodium citrate. Plasma was prepared by centrifugation at 1,800 g for 15 minutes at room temperature. The plasma was separated and centrifuged again to ensure that platelet-poor plasma was collected. Samples were aliquoted and stored at −70°C until required.

Pooled normal plasma was prepared from 20 normal male donors. Equal volumes of plasma from each donor were pooled, mixed, aliquoted and stored at −70°C until required. Pooled normal plasma was designated to contain 1 U/mL TFPI activity.

TFPI activity assay
TFPI activity was measured using an amidolytic assay based on a previously described method. Prior to the assay, test or standard plasmas were heated at 56°C for 15 minutes, placed on melting ice for 2 minutes then centrifuged for 15 minutes at 1,800 g. Diluted test or standard plasma (25 µL) was incubated with 100 µL of a reaction mixture, which contained equal amounts of recombinant TF (Innovin®, Dade Behring, Marburg, Germany) (diluted 1/40 in TFPI assay buffer containing 0.15 mol/L NaCl, 0.05 mol/L Tris, 0.01 mol/L trisodium citrate, 0.1% normal serum albumin and 0.02% polybrene, pH 8.0), 0.03 U/mL FVII (Enzyme Research Laboratories, Southbend, USA), 0.025 U/mL FX (Sigma Chemical Company, St Louis, USA), and 0.05 mol/L CaCl₂, in U-bottomed microtiter plates (Australian Biosearch, Perth, Australia). After incubation for 20 minutes at 37°C, 50 µL of substrate mixture, containing 0.4 U/mL FX and 2.7 mmol/L synthetic peptide substrate (S-2222; Chromogenix, Milan, Italy), were added and incubated at 37°C for a further 30 minutes. The reaction was stopped by the addition of 50 µL of 50% acetic acid and the absorbance read at 405 nm using a Multiskan Acent microtiter plate spectrophotometer. The assay was standardized using pooled normal plasma from 20 healthy male donors.

IgG isolation
IgG fractions were isolated from plasma by affinity purification using a protein G column according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Uppsala, Sweden). Samples were diluted 1 in 5 in 0.02 mol/L phosphate-buffered saline (PBS) pH 7.4 and pushed slowly through the column using an attached syringe. After washing the column with 10 mL of 0.02 mol/L PBS pH 7.4, IgG was eluted with 5 mL of 0.1 mol/L glycine-HCl pH 2.8 and collected as 1 mL aliquots into 100 µL of 1.0 mol/L tris-HCl pH 9.0. The absorbance of each aliquot was measured at 280 nm and the two aliquots with the highest absorbance and 0.5 mL of the third highest aliquot were pooled. Buffer exchange was performed using PD 10 desalting columns (Amersham Pharmacia Biotech, Uppsala, Sweden), equilibrated with 15mL of TFPI assay buffer pH 8.0 or 0.02 mol/L PBS pH 7.4. Isolated IgG (2.5 mL) was passed through the desalting column and eluted with 3.5 mL of the appropriate buffer. The concentration of IgG was determined by measuring the absorbance of the pooled aliquots with a 1 cm light path and an extinction coefficient of Eᵣ₁₀₀₀ = 13.6, and stored at −70°C.

Preparation of pooled normal IgG
Following isolation (as described above) from 22 normal controls, IgG fractions were adjusted to a concentration of 500 µg/mL with PBS pH 7.4 and an equal volume of each fraction pooled, aliquoted and stored at −70°C until required.

Determination of anti-TFPI activity
IgG fractions (25 µg/mL) were mixed with pooled normal plasma for 30 minutes at 37°C. The IgG and pooled normal plasma mixtures were then re-assayed for TFPI activity. Anti-TFPI activity was designated as the difference between TFPI activity in the IgG-pooled normal plasma mixture and the control (buffer/pooled normal plasma; 1 U/mL).

Assay of thrombin generation
A chromogenic assay based on the method of Sheng et al. was used to determine the effect of IgG fractions from controls and aPS subjects on TF-induced in vitro thrombin generation. Plasma was initially defibrinated by heating at 53°C for 20 minutes, incubation on melting ice for 2 minutes and centrifugation at 3,000 g for 10 minutes. Thromboplastin (25 µL, diluted 1/10 in 0.9% NaCl) and 50 µL of defibrinated plasma (diluted 1/2 in 0.02 mol/L PBS pH 7.4 or 25 µg/mL IgG/antibody) were incubated for 10 minutes at 37°C. Chromozyme TF substrate (Roche Diagnostics, Sydney, Australia) was diluted in 0.9% NaCl to a concentration of 0.95 mmol/L and 50 µL added to each well. Following addition of 30
mmol/L CaCl₂ (50 µL), the absorbance was measured at 405 nm every two minutes until the peak absorbance was obtained. Each sample was measured for background absorbance by substituting TF with 25 µL of 0.9% NaCl. At each time point the absorbance of the background wells was subtracted from the absorbance of the corresponding test wells. This resulted in the final corrected absorbance for each sample assessed. Thrombin generation (%) was determined by dividing the absorbance at 1/2max of the test mixture by the absorbance at 1/2max of a blank (containing 0.9% NaCl instead of test or control IgG).

A sheep anti-rabbit TFPI antibody (a gift from Dr P. Tipping, Monash University, Australia) and pooled IgG from normal controls were used as controls.

Statistics
Results were analyzed using StatViewSE+Graphics for Macintosh (Abacus Concepts, Berkeley, USA). Results are expressed as mean ± standard deviation (SD) for TFPI activity or median (inter-quartile range) for other data sets. Between group differences were assessed using Student’s t-test for normally distributed data (TFPI activity) or the Wilcoxon signed rank test for non-parametric data. Correlations between sets of data was determined using Spearman’s correlation co-efficient. p values < 0.05 were considered to be statistically significant.

Results
TFPI activity
TFPI activity (mean±SD) in controls (1.13±0.25 U/mL) was significantly lower than in aPS subjects (1.30±0.42 U/mL) (p < 0.05) (Figure 1). Two aPS subjects had TFPI activity below the lower 95% confidence interval (< 0.63 U/mL) of the normal range (0.57 and 0.53 U/mL, respectively). Seven aPS subjects had TFPI activity above the upper 95% confidence interval (> 1.63 U/mL) of the derived normal range.

Anti-TFPI activity
Anti-TFPI activity was detected in 3/29 (10%) controls (0.01, 0.02 and 0.02 U/mL) and in 37/57 (65%) aPS subjects [median = 0.03 (0.02 – 0.06 U/mL)] (Figure 2). Anti-TFPI activity was significantly greater in aPS subjects than in controls (p = 0.0001). There was no correlation between TFPI activity and anti-TFPI activity in either control or subject cohorts (p > 0.05).

TF-induced thrombin generation
The standard curve for the detection of in vitro TF-induced thrombin was sigmoidal in shape (Figure 3), as previously demonstrated.13 A sheep anti-rabbit TFPI antibody induced a concentration-dependent increase in thrombin generation (Figure 4). Pooled IgG from normal donors demonstrated no effect on thrombin generation (Figure 4). The effect on TF-induced thrombin generation by IgG fractions from 10 controls and 23 aPS subjects was variable [100.5% (97.0 – 102.0%) vs 98.0% (89.5 – 105.5%)] (p > 0.05) (Figure 5). The effect of IgG fractions from 23 of 57 aPS subjects tested on thrombin generation was positively correlated (p = 0.356) with anti-TFPI activity (Figure 6), but this was not statistically significant (p = 0.09). There was no correlation between the effect of IgG on in vitro TF-induced thrombin generation and TFPI activity in either control (p > 0.05) or subject cohorts (p > 0.05).
Venous or arterial thrombosis is a frequent clinical manifestation of aPS, though causal mechanisms remain to be elucidated. It is probable that the development of a hypercoagulable state is a consequence of multiple significant abnormalities and/or imbalances of hemostatic mechanisms, including the TF pathway. TFPI activity levels were initially demonstrated to be higher in aPS subjects than in controls. Though variable levels of TFPI activity (and antigen) have been reported in aPS subjects, recent experimental evidence supports up-regulation of the TF pathway as an important factor in the development of hypercoagulability in aPS. Indeed, increased TFPI activity in the plasma of aPS subjects probably represents increased endothelial cell secretion of TFPI. This may be a protective mechanism in response to the direct influence of aPL, which have been demonstrated to induce an increase in TF-like activity in monocytes and endothelial cells.

The presence of anti-TFPI activity was confirmed in the cohort of aPS subjects used in this study. Interestingly, anti-TFPI activity was demonstrated in a greater proportion of subjects compared to an earlier study (65% vs 15%). It is unclear why there is a large discrepancy between these results, as the methods to measure anti-TFPI activity were essentially the same. A relatively small sample size in each study, or the use of fresh pooled nor-

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**Discussion**

Venous or arterial thrombosis is a frequent clinical manifestation of aPS, though causal mechanisms remain to be elucidated. It is probable that the development of a hypercoagulable state is a consequence of multiple significant abnormalities and/or imbalances of hemostatic mechanisms, including the TF pathway. TFPI activity levels were initially demonstrated to be higher in aPS subjects than in controls. Though variable levels of TFPI activity (and antigen) have been reported in aPS subjects, recent experimental evidence supports up-regulation of the TF pathway as an important factor in the development of hypercoagulability in aPS. Indeed, increased TFPI activity in the plasma of aPS subjects probably represents increased endothelial cell secretion of TFPI. This may be a protective mechanism in response to the direct influence of aPL, which have been demonstrated to induce an increase in TF-like activity in monocytes and endothelial cells.

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Anti-TFPI activity and thrombin generation in aPS.

The range of anti-TFPI activity in aPS subjects was 0.01 - 0.10 U/mL, reflecting variability in the strength of the interference. Three (10%) of 29 normal controls also demonstrated anti-TFPI activity, although levels (0.01 – 0.02 U/mL) were lower than those detected in the majority of aPS subjects. This was not an unexpected observation as laboratory markers of aPS, lupus anticoagulants (LA) and anticardiolipin antibodies (aCL), have been reported in the plasma of normal individuals.20-23 Anti-TFPI autoantibodies have also been reported in a small percentage of normal individuals.23

The primary aim of this study was to investigate the influence of IgG fractions from aPS subjects on in vitro TF-induced thrombin generation. The assay, based on a previously published method,13 measures the effect of antibody, IgG or plasma in a chromogenic substrate assay of thrombin generation. It detects both LA and β2GPI dependent aCL antibodies and probably has greater specificity for aPS than currently available diagnostic tests.13 It was hypothesized in the present study that IgG fractions obtained from the plasma of aPS subjects which contained anti-TFPI activity would increase in vitro TF-induced thrombin generation. Physiological concentrations of TFPI have been demonstrated to reduce the rate of thrombin generation,24 thus it was predicted that inhibition of TFPI activity would increase the rate of thrombin generation.

It was initially demonstrated in control experiments that a sheep anti-rabbit TFPI antibody increased in vitro TF-induced thrombin generation in a concentration-dependent manner and that pooled normal IgG had no effect on thrombin generation in the same system. These controls confirmed that changes to thrombin generation were due to interference of TFPI activity by a component of the IgG fractions.

The effect of IgG fractions from normal controls on thrombin generation demonstrated a narrow inter-quartile range of 97-102%. This variation may be accounted for by the incidental presence of aPL in the IgG fractions of apparently healthy subjects24 and might therefore influence thrombin generation. The effect of IgG fractions from aPS subjects, however, was more variable, with a wider inter-quartile range (89.5% – 105.5%) and low and high values of 49% and 131%, respectively, indicating a variety of influences from IgG fractions on TF-induced thrombin generation. In particular, increased thrombin generation may be due to interference with TFPI activity, for example by anti-TFPI antibodies and/or other cross-reacting aPL, reflecting increased hypercoagulability and thrombotic risk.

Anti-TFPI activity in aPS subjects was weakly correlated with the effect of IgG fractions on TF-induced in vitro thrombin generation (r = 0.356). Interestingly, increased thrombin generation (above the 75% percentile of the normal cohort; 102%) and anti-TFPI activity were reported in 5 of the 23 aPS subjects tested. The presence of anti-TFPI activity in the plasma of these subjects could contribute significantly to an increased thrombotic risk. A larger cohort of subjects may demonstrate a significant positive correlation between anti-TFPI activity and the resulting influence of IgG fractions (or purified anti-TFPI autoantibodies from these subjects) on TF-induced in vitro thrombin generation.

Low TF-induced thrombin generation (below the 25th percentile of the normal cohort; 97%) was reported in 10 of the 23 subjects tested. This is probably due to the presence of LA, which paradoxically interfere with in vitro phospholipid-dependent coagulation tests [including the thrombin generation assay used in this study]13 by prolonging clotting times, thus reflecting decreased, rather than increased thrombin generation. Each of the 10 subjects tested was positive for LA. It is also interesting to note that the 5 IgG fractions that generated increased TF-induced thrombin generation were also positive for LA, indicating that the overall strength of anti-TFPI activity was greater than the influence of LA. The model used in this study therefore accounts for the overall capacity of anti-TFPI activity in IgG fractions, which potentially contain LA, aCL and other entities, to influence thrombin generation, rather than the influence of anti-TFPI antibodies alone.

This study has confirmed the presence of anti-TFPI activity in aPS subjects, demonstrating that IgG fractions generate variable influences on TF-induced thrombin generation and associated anti-TFPI activity with increased thrombin generation. Whether the anti-TFPI activity demonstrated in this in vitro system represents the presence of in vivo anti-TFPI autoantibodies, other aPL that interfere with TFPI activity such as anti–β2GPI26 or a combination of both, is yet to be determined. The overall contribution of these factors and others, such as the influence of aPL on TF expression or secretion, may be significant in altering the balance of the TF pathway and the development of the clinical manifestations associated with aPS.

MA is the author taking primary responsibility for this paper. MA, LB, PS, JT, RB and RO each contributed to the conception and design of the study, data analysis, interpretation of results, and preparation and review of the manuscript. LB and PS performed the experiments and collected the data. The authors reported no potential conflicts of interest.

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