

Anti-tissue factor pathway inhibitor activity in patients with primary antiphospholipid syndrome

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Summary. The association between antiphospholipid antibodies and an increased risk of thrombosis in antiphospholipid syndrome (aPS) patients is probably caused by numerous mechanisms, including the effects of antibodies to phospholipid-binding proteins such as β_2 -glycoprotein I and prothrombin. In this study, we investigated the inhibition of tissue factor pathway inhibitor (TFPI) in 33 patients with primary antiphospholipid syndrome (PAPS). TFPI was measured in PAPS patients using an amidolytic assay, dependent on the generation of activated factor X (Fxa), and this was compared with 55 healthy subjects. Functional levels of TFPI (mean \pm SD) were significantly lower in PAPS patients (0.89 ± 0.37 U/ml) than the control group (1.05 ± 0.15 U/ml) ($P = 0.02$). The difference was caused by a subset of five patients who had TFPI levels below the lower 99% confidence interval of the

normal reference range, representing increased FXa generation in the assay system. IgG fractions were isolated from these five patients and five control subjects, then incorporated into normal plasma to measure FXa generation in the TFPI assay system. FXa generation was increased when polyclonal rabbit anti-human TFPI IgG ($P < 0.0001$) or PAPS IgG ($P = 0.0001$) were added to normal plasma, demonstrating inhibition of TFPI. The apparent anti-TFPI activity demonstrated in the five subjects with PAPS in this study may represent a significant new mechanism for thrombosis in patients with aPS, as it implies that increased tissue factor FVIIa-mediated thrombin generation might occur.

Keywords: TFPI, antiphospholipid syndrome, anti-TFPI activity, factor Xa, thrombosis.

The antiphospholipid syndrome (aPS) is defined as the association of circulating autoimmune antiphospholipid antibodies (aPL) with arterial or venous thrombosis, recurrent fetal loss or thrombocytopenia (Hughes *et al*, 1986; Harris *et al*, 1987; Love & Santoro, 1990; Wilson *et al*, 1999). aPL are directed against phospholipid-binding proteins including β_2 -glycoprotein (β_2 GPI) and prothrombin, and include lupus anticoagulant (LA) and anticardiolipin antibodies (aCL) (McNeil *et al*, 1990). Other, as yet unidentified, target antigens may be involved in aPS, and could include components of the tissue factor (TF) pathway of coagulation. Recent evidence suggests that upregulation of the TF pathway may be important in contributing to the hypercoagulability seen in aPS (Amengual *et al*, 1998). Reports by Adams *et al* (1995) who described decreased tissue factor pathway inhibitor (TFPI) activity in patients with lupus anticoagulants, and Saleminck *et al* (2000) who reported enhanced FXa generation in three aPS patients in the presence of β_2 GPI antibodies to β_2 GPI and TFPI, support this evidence.

TFPI regulates the initiation of the tissue factor pathway by forming a quaternary complex (TF–FVIIa–FXa–TFPI) after the exposure of TF to the circulation (Broze *et al*, 1988). Upregulation of the TF pathway could potentially be caused by decreased TFPI activity or by an antibody to TFPI, contributing to the hypercoagulability in patients with aPS. In this paper, we report the presence of anti-TFPI activity in five subjects with primary antiphospholipid syndrome (PAPS), which could contribute to the thrombotic events in these patients.

PATIENTS AND METHODS

Patients and samples

Venous blood was collected with minimal stasis from 33 well characterized subjects with PAPS (Exner *et al*, 1991; Greaves *et al*, 2000). Blood was collected into empty tubes for the preparation of serum, or Vacutainers (Becton Dickinson, Oxford, UK) containing 0.105 mol/l tri-sodium citrate. Plasma was prepared by centrifuging at $2000 g$ for 15 min at room temperature. The plasma was separated and centrifuged again to ensure platelet depletion. Serum

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and double-spun plasma samples were stored in aliquots at -70°C until required.

Antiphospholipid antibody testing

Lupus anticoagulant. LA was detected according to standard criteria (Greaves *et al*, 2000) using a commercial kit for the dilute Russell's Viper Venom Time, with a platelet neutralization procedure (Unicorn Diagnostics, London, UK).

Anticardiolipin antibodies. IgG and IgM antibodies to cardiolipin were measured in serum samples using an enzyme-linked immunosorbent assay (ELISA) technique using microtitre plates (Polysorp, Nunc, Life Technologies, Paisley, UK) coated with cardiolipin (bovine heart, Sigma-Aldrich, Poole, UK). Assays were standardized using standard sera calibrated against the appropriate International Reference Material (Harris & Hughes, 1987).

Anti- β_2 GPI antibodies. Anti- β_2 GPI IgG was measured by ELISA, using a commercial kit (Diastat anti β_2 GPI, Axis-Shield Diagnostics, Dundee, UK) based on a method previously developed in our laboratory (McNally *et al*, 1995). Anti- β_2 GPI IgM was assayed by an adaptation of the IgG kit components to accommodate the detection of IgM antibodies by using a μ -specific conjugated secondary antibody. Assays were standardized using serum with a known high concentration of antibody.

Antiprothrombin antibodies. Antiprothrombin IgG and IgM were analysed by ELISA using μ -irradiated microtitre plates (Maxisorp, Nunc, Life Technologies, Paisley, UK) coated overnight at 4°C with $10\ \mu\text{g/ml}$ human prothrombin (Enzyme Research Laboratories, Swansea, UK) in phosphate-buffered saline (PBS) ($0.01\ \text{mol/l}$ phosphate, $0.145\ \text{mol/l}$ NaCl, pH 7.2). Blocking was performed using PBS containing 0.1% Tween 20 and 1% bovine serum albumin (BSA; Sigma-Aldrich, Poole, UK) for 1 h at room temperature. Plates were then washed once using PBS containing 0.1% Tween 20. A standard curve was prepared from reference plasma with a known high-aPT activity. Test samples were diluted 1:50 in PBS containing 0.1% Tween 20 and 1% BSA. Next, $100\ \mu\text{l}$ of standard or test was added to duplicate wells for 1 h at room temperature. After three washes, $100\ \mu\text{l}$ of alkaline phosphatase-conjugated anti-human IgG or IgM was added for 1 h. *p*-Nitrophenyl phosphate ($100\ \mu\text{l}$; Sigma-Aldrich, Poole, UK) in diethanolamine buffer (pH 9.8) was added and incubated at 37°C for colour development, which was stopped using $3\ \text{mol/l}$ NaOH, and the absorbance read at 405 nm.

All assays were validated by the inclusion of internal quality control samples of known activity. The cut-off point for the upper limit of normal was determined as the geometric mean + 95% Confidence Interval (CI) of values obtained in at least 30 apparently healthy adults.

TFPI activity assay

TFPI was measured using an amidolytic assay based on the method of Sandset *et al* (1987). Briefly, $25\ \mu\text{l}$ of diluted test or standard plasma was incubated with $100\ \mu\text{l}$ of reaction mixture containing equal amounts of recombinant TF (diluted 1/40) (Innovin, Dade Behring, Marburg, Germany),

$0.013\ \text{U/ml}$ FVII (Sigma-Aldrich), $0.03\ \text{U/ml}$ FX (Enzyme Research Laboratories) and $0.075\ \text{mol/l}$ CaCl_2 , in flat-bottomed microtitre plates (Alpha Laboratories, Eastleigh, UK). After incubation for 10 min at 37°C , $50\ \mu\text{l}$ of substrate mixture containing FX ($0.6\ \text{U/ml}$) and an amidolytic substrate for FXa (S-2222, $2.7\ \text{mmol/l}$; Chromogenix, Mölndal, Sweden) was added and incubated at 37°C for a further 30 min. The reaction was stopped by the addition of $50\ \mu\text{l}$ of 50% acetic acid. The TFPI activity assay was standardized using Immuno Reference Plasma 100% (Technoclone, Vienna, Austria). Results were derived from the mean of duplicate tests for all assays. The reference range was defined as the mean ± 3 standard deviations.

Protein G chromatography

The IgG fraction was isolated from plasma by affinity purification on a Protein G column according to the manufacturer's instructions (Pharmacia Biotech, St Albans, UK). Samples were diluted 1 in 5 in PBS pH 7.4 and applied to a pre-equilibrated Protein G column. Bound IgG was eluted with glycine buffer, pH 2.8 and collected in 4 ml fractions containing $0.6\ \text{ml}$ $1.0\ \text{mol/l}$ Tris. Fractions containing protein were pooled, dialysed to PBS and concentrated using an Amicon concentrator and a PM30 membrane. The protein concentration was determined by measuring the absorbance with a 1 cm light path and an extinction coefficient of $\text{E}^{1\%}_{1\text{cm}} = 13.6$, aliquoted and stored at -70°C .

IgG mixing studies

IgG fractions were isolated from the five PAPS subjects with increased FXa activity in the functional TFPI assay, and five normal controls. The fractions were mixed with normal plasma for 30 min at 37°C to a final concentration of $10\ \mu\text{g/ml}$ and then assessed for functional TFPI activity. A rabbit anti-human polyclonal TFPI antibody (American Diagnostica, Greenwich, USA) was used in this system as a control to determine loss of TFPI activity in the normal plasma.

Statistical analysis

Between-group differences in functional TFPI were analysed using the student's two tailed *t*-test for unpaired data. *P*-values < 0.05 were considered statistically significant.

RESULTS

Patients – clinical and laboratory details

Patients in this study were well characterized and did not have acute ongoing thrombotic events. These included 25 female and 8 male patients, mean age 47.8 ± 14.4 years (range 25–74 years). Twenty-six (79%) patients had a clinical history of thrombosis (58% venous; 21% arterial), 20 (61%) were unequivocally LA-positive, 20 (61%) had anti- β_2 GPI IgG/M antibodies, 16 (48%) antiprothrombin IgG/M antibodies and 25 (76%) anticardiolipin IgG/M antibodies.

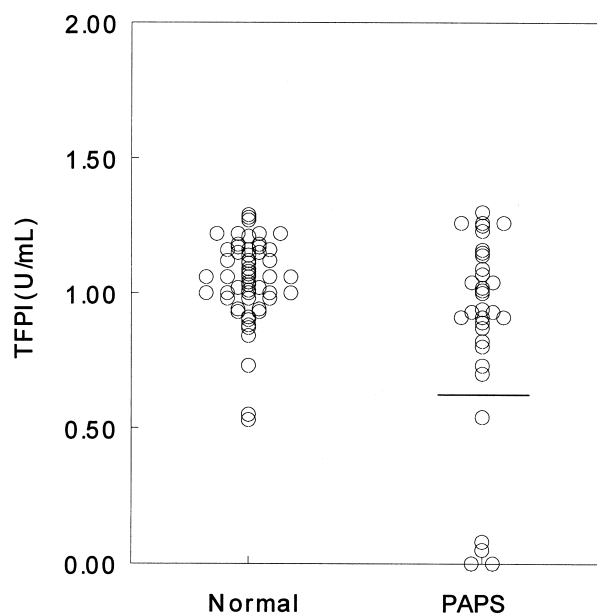


Fig 1. Functional TFPI in normal and PAPS subjects. Bar indicates lower 99% confidence interval of the normal reference range.

TFPI levels

The laboratory normal range for the functional TFPI assay was established using plasma from 55 healthy subjects (37 men, 18 women; mean age = 44.2 years) with no apparent haemostatic abnormality. The reference range was 0.60–1.50 U/ml (mean = 1.05 U/ml; standard deviation = 0.15 U/ml) (Fig 1).

Mean functional TFPI levels were 0.89 ± 0.37 U/ml in 33 subjects with PAPS, which was significantly lower than levels in the normal subjects ($P = 0.02$) (Fig 1). The lower functional TFPI in the subject group was caused by a

subpopulation of samples ($n = 5$), which generated increased FXa activity (below the lower 99% confidence interval of the reference range). Three of these patients were receiving warfarin and aspirin, one was receiving aspirin only and one was receiving warfarin only as part of thromboprophylaxis because of previous events. No patient received heparin as part of their treatment.

Table I summarizes the major clinical and laboratory details of the five patients that demonstrated increased FXa activity in the functional assay for TFPI.

TFPI inhibition

TFPI activity in normal plasma was 100% inhibited after 30 min at 37°C by the addition of a polyclonal rabbit anti-human TFPI antibody (10 µg/ml). When the IgG fractions from the five selected patients was mixed with normal plasma, the mean TFPI activity was reduced from 0.99 U/ml to 0.72 U/ml ($P = 0.0001$) (Fig 2). In each case, this represented a reduction of greater than 25% TFPI activity, and the effect was demonstrated on two separate occasions. When IgG (0–20 µg/ml) from one of the five PAPS subjects with low TFPI activity was added to normal plasma, TFPI activity was reduced in a concentration-dependent manner (Fig 3). Control IgG (final concentration = 10 µg/ml) collected from five normal subjects demonstrated less than 1% reduction (0.98 U/ml) in TFPI activity when mixed with normal plasma.

DISCUSSION

There have been varying reports in the literature on TFPI levels in patients with aPS. TFPI antigen levels have been reported to be both higher (Amengual *et al.*, 1998; Wakita *et al.*, 1999), and lower (Kiraz *et al.*, 1999), compared with the control groups. Although these variations have yet to be

Table I. Comparison of laboratory characteristics of PAPS subjects with normal ($n = 28$) and decreased ($n = 5$) TFPI activity in the functional assay for TFPI.

PAPS subjects	Normal TFPI activity ($n = 28$)	Decreased TFPI activity ($n = 5$)
Sex (M:F)	6:22	3:2
Arterial thrombosis	3 (11)	4 (80)
Venous thrombosis	19 (68)	0 (0)
↑ Anti-β ₂ GPI		
IgG	14 (50)	4 (80)
IgM	9 (32)	1 (20)
↑ Antiprothrombin		
IgG	7 (25)	1 (20)
IgM	23 (82)	5 (100)
↑ Anticardiolipin		
IgG	20 (71)	2 (40)
IgM	8 (29)	3 (60)
Lupus-anticoagulant positive	17 (61)	3 (60)
TFPI (U/ml)	1.05 ± 0.17	0.13 ± 0.23

Percentages are given in parenthesis.

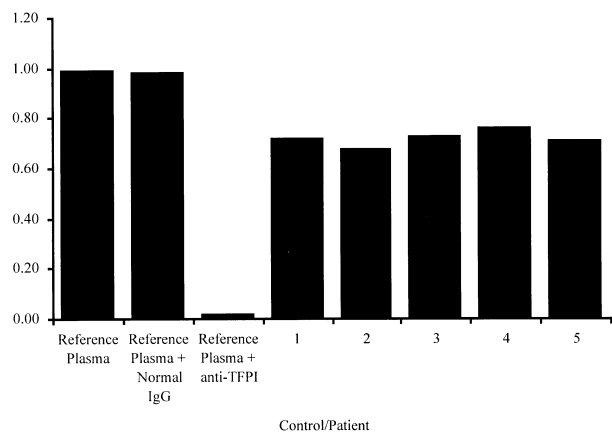


Fig 2. Functional TFPI levels in normal plasma with the incorporation of the IgG fraction from five patients with PAPS. Normal plasma = 0.99 U/ml. Normal plasma + normal IgG = 0.98 U/ml. Normal plasma + anti-TFPI = 0.02 U/ml. Columns 1–5 represent functional TFPI activity in normal plasma mixed with IgG from patients with PAPS (mean = 0.72 U/ml; $P = 0.02$).

explained, it is feasible that an upregulation of coagulation, with increased levels of both TFPI and TF, could contribute to thrombosis in patients with aPS. Alternatively, if there is an imbalance between TF and TFPI levels, an increased risk of thrombosis may result owing to low levels of TFPI in the presence of normal or elevated TF.

Decreased TFPI activity in patients with LA has previously been demonstrated in an *in vitro* assay system (Adams *et al*, 1995; Adams & Oosttryck, 1997). The increased FXa generated in this system was abolished with the addition of excess amounts of thromboplastin. This inferred that LA preferentially targeted the phospholipid component of the assay, and was associated with decreased TFPI inhibition of TF–FVIIa–FXa. It was concluded that both the amount and source of thromboplastin used in this particular system affected the measurement of functional TFPI in patients with LA.

Subsequent reports have implicated the inhibition of TFPI anticoagulant activity by strong lupus anticoagulants (Jacobsen *et al*, 1999), and described enhanced FXa generation in three patients with aPS that was dependent on the presence of β_2 GPI, antibodies to β_2 GPI and TFPI (Salemink *et al*, 2000). Perhaps of most significance is a recent report on the presence of anti-TFPI antibodies in patients with aPS (Cakir *et al*, 2000). These antibodies were associated with stroke and arterial thrombosis, but not with other classical clinical features of aPS such as venous thrombosis.

In this study of a cohort of 33 PAPS patients, we report increased FXa generation (and, therefore, apparent TFPI reduction), as measured in a functional assay for TFPI. The statistical difference between PAPS patients and normal volunteers was caused by a small ($n = 5$) subset of the patient population. When these five patients were removed from the statistical analysis, there was no significant difference in functional TFPI levels. Four of the five patients

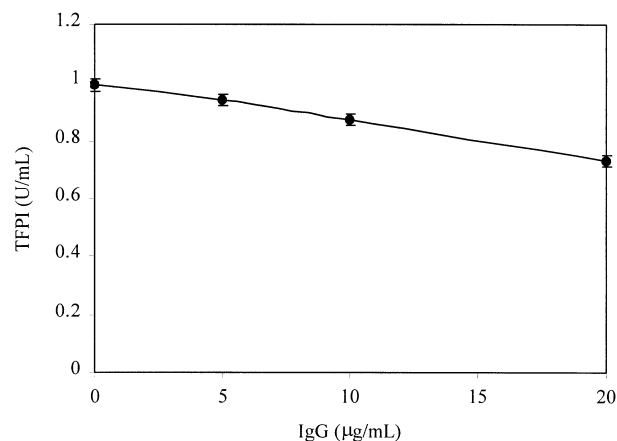


Fig 3. Effect of IgG concentration from a PAPS subject with low TFPI activity in normal plasma.

had a history of arterial disease, but none had venous thromboembolism, in keeping with the results of Cakir *et al* (2000).

We also demonstrated inhibition of TFPI activity after the addition of IgG fractions from the same five PAPS patients to normal plasma. The reduction in TFPI activity was consistently greater than 25%, and was reproduced on two separate occasions. The increased generation of FXa is evidence of anti-TFPI activity in the IgG fraction from the selected PAPS patients.

The anti-TFPI activity demonstrated in this study is further evidence of an autoantibody to TFPI. Identification of a specific pathological anti-TFPI antibody will probably be confirmed with continued screening of IgG and/or IgM fractions from aPS patients, and the use of affinity chromatography for anti-TFPI. IgM anti-TFPI antibodies may be present in blood samples from PAPS patients and could explain some of the observed discrepancies between TFPI activity in patient plasma and the degree of inhibition of TFPI in mixing experiments of normal plasma and patient IgG. The presence of an anti-TFPI antibody in the plasma from patients with aPS patients would provide an explanation to the discrepant results of earlier reports (Adams *et al*, 1995; Adams & Oosttryck, 1997). Anti-TFPI antibodies may contribute towards the overall pathophysiological mechanisms of thrombosis in aPS patients. Indeed, in this study all five patients that demonstrated anti-TFPI activity also had a history of arterial thrombosis and/or stroke. Future work will be required to determine the incidence, clinical significance and the association with other antiphospholipid antibodies.

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