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Tissue factor pathway inhibitor anticoagulant activity: risk for venous thrombosis and effect of hormonal state

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Summary

Full-length tissue factor pathway inhibitor (TFPI) is assumed to be biologically more important than truncated TFPI because of its stronger anticoagulant effect in the diluted prothrombin time (dPT) assay. We have developed a dPT-based assay for TFPI anticoagulant activity. Here, we report the effect of hormonal state on TFPI anticoagulant activity and whether TFPI anticoagulant activity assesses the risk for deep-vein thrombosis (DVT) better than conventional TFPI assays. We undertook a case-control study of 474 patients with DVT and 474 controls and compared the odds ratio (OR) for DVT for those with low TFPI anticoagulant activity with the ORs obtained for TFPI free and total antigen, and TFPI chromogenic substrate activity. Hormonal state affected clot time in dPT, but this effect was ameliorated by anti-TFPI antibodies. Low TFPI anticoagulant activity gave an OR of 1·5 (0·97–2·1) for DVT, similar to the ORs obtained with conventional TFPI assays, ranging from 1·2 to 1·4. Individuals low in both the activity assays obtained an OR of 5·9 (1·7–20). We concluded that the effect of hormonal state on dPT was mediated through TFPI, and a dPT-based assay of TFPI anticoagulant activity did not assess the risk for DVT better than conventional TFPI assays.

Keywords: deep-vein thrombosis, diluted prothrombin time, thrombophilia, tissue factor, tissue factor pathway inhibitor.

Tissue factor pathway inhibitor 1 (TFPI) is the physiological inhibitor of blood coagulation initiated by tissue factor (TF). There is now convincing evidence that TFPI plays a key role in regulating blood coagulation *in vivo*. First, TFPI gene knock-out mice suffered abnormal intrauterine development and death (Huang *et al*, 1997). Secondly, immunodepletion of TFPI in rabbits significantly decreased the threshold by which TF may trigger coagulation and induce disseminated intravascular coagulation (Sandset *et al*, 1991a,b). Thirdly, high doses of recombinant TFPI protected against various experimentally induced thrombotic disorders (Holst *et al*, 1993; Kaiser & Fareed, 1996; Bajaj & Bajaj, 1997). Moreover, it has recently been reported that low levels of TFPI is a risk factor for both venous and arterial thrombosis (Dahm *et al*, 2003; Morange *et al*, 2004). Therefore, it is intriguing that familial TFPI mutations associated with low levels of TFPI and increased risk of thrombosis have not yet been convincingly reported (Sandset & Bendz, 1997).

In contrast to other coagulation inhibitors, e.g. antithrombin and protein C, the intravascular distribution of TFPI *in vivo* is rather complex. Most intravascular TFPI is free full-length TFPI, which is associated with the vascular endothelium and which may be released by heparin and other negatively charged polyanions (Sandset *et al*, 2000). A small amount of free full-length TFPI is also found in circulating blood, but plasma contains mostly truncated forms of TFPI, which is to a great extent, bound to lipoproteins (Novotny *et al*, 1989; Lindahl *et al*, 1991; Hansen *et al*, 1994). The role of different forms of TFPI are not well known, but as full-length TFPI exerts a much stronger anticoagulant effect in diluted prothrombin time (dPT)-based assays than truncated and lipoprotein-associated TFPI (Lindahl *et al*, 1991; Nordfang *et al*, 1991; Hansen *et al*, 1995), it has been suggested that full-length TFPI is biologically more important *in vivo*. The complex distribution and functional aspects of TFPI raises the important question of how to detect TFPI deficiency and

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functional defects *in vivo*, and it has been speculated that a dPT-based assay of TFPI might reveal TFPI deficiencies that other assays fail to detect (Sandset & Bendz, 1997).

Currently published assays detect free full-length TFPI (TFPI-free antigen) or all forms of TFPI (TFPI total antigen) in plasma (Table I). Functional assays involve prolonged incubation of TFPI in plasma with TF, factor VIIa (FVIIa) and factor Xa (FXa), which allows the formation of inactive TFPI/FXa/TF/FVIIa inhibitory complexes, and residual TF/FVIIa catalytic activity is determined with a substrate, e.g. FX and a chromogenic substrate or tritium-labelled factor IX. Such functional assays correlated strongly with TFPI total antigen levels (Bendz *et al*, 2000; Dahm *et al*, 2005) and may be rather insensitive to functional defects of TFPI.

We have recently developed a novel assay of TFPI anticoagulant activity (Dahm *et al*, 2005), which is based on the ability of TFPI to prolong the clotting time after initiation of coagulation with a low concentration of TF, i.e. a dPT assay. In this assay, dPT is run both in the presence and in the absence of TFPI neutralising antibodies. The shortening of clotting times in the presence of TFPI antibodies reflects TFPI anticoagulant activity. The TFPI anticoagulant activity assay is specific for full-length TFPI and insensitive to truncated TFPI. In the present study, we have evaluated the role of TFPI anticoagulant activity as a risk factor for the development of deep-vein thrombosis (DVT) and studied the effect of hormonal state on dPT and TFPI using blood samples collected in the Leiden Thrombophilia Study (LETS).

Patients and methods

Leiden thrombophilia study – study population and blood sampling

The design of the LETS case–control study has previously been described in detail (Koster *et al*, 1993). Briefly, 474 patients with an objectively diagnosed first time DVT were compared with 474 sex- and age-matched controls. In a previous report, we reported TFPI free and total antigen and TFPI chromogenic substrate activity data in 473 patients and 473 control subjects (Dahm *et al*, 2003). The current report concerns data on TFPI anticoagulant activity in 452 cases and 439 controls where blood samples were available for analysis. The patients were selected from three anticoagulation clinics in the Netherlands. The controls were acquaintances of the patients or partners of other patients. Patients with known malignancies were excluded and all patients were younger than 70 years of age. The Leiden University Medical Centre ethics committee approved the study protocol, and all participants gave informed consent according to the Declaration of Helsinki. Blood samples were taken between 6 and 56 months after the thrombosis was diagnosed, hence most patients had finished their anticoagulant treatment at the time of blood sampling. Whole blood (0·9 v) was collected from the antecubital vein into Sarstedt Monovette tubes (Sarstedt, Nümbrecht, Germany) containing 0·106 mol/l of trisodium citrate (0·1 v). Plasma was prepared by centrifugation for 10 min at 2000 ***g*** at room temperature and stored in aliquots at −70°C until assayed.

TFPI assays

The 'TFPI anticoagulant activity' assay was based on the assay of dPT in the presence and the absence of neutralising TFPI antibodies (anti-TFPI IgG) as reported in detail elsewhere (Dahm *et al*, 2005). Briefly, 32 μl of plasma was incubated with 8 μl Tris-buffered saline or 8 μL anti-TFPI IgG and incubated for 4–6 min at room temperature. Thirty microlitres of TF (Innovin4, diluted 1/200; Dade Behring, Marburg, Germany) was then added and incubated for 3 min at 37°C. Finally, 30 μl 35 mmol/l $CaCl_2$ was added and the time to clotting recorded with the Amelung KC4 coagulometer (Trinity Biotech, Bray, Ireland). Clotting time in the absence of anti-TFPI IgG was approximately 75 s, which was reduced by approximately 7–8 s in the presence of anti-TFPI IgG in normal reference plasma. Test results were expressed as a TFPI anticoagulant ratio of the dPT recorded in the absence of anti-TFPI IgG on the dPT in the presence anti-TFPI IgG. Both dPTs as well as the TFPI anticoagulant ratio were normalised against results obtained with the normal reference plasma to minimise the effects of variations in assay conditions, e.g. day-to-day and batch-to-batch variations or reagents. Thus, a low normalised dPT meant a short dPT, while a high normalised dPT meant a

Table I. Overview of the assays of circulating TFPI.

	TFPI assay	Measures what
Strongly correlated	TFPI-free antigen	Amount of full-length TFPI
	TFPI anticoagulant activity	The ability of full-length TFPI to inhibit FXa. Insensitive to truncated TFPI
Not strongly correlated	TFPI chromogenic substrate activity	The ability of the total TFPI, i.e. full-length and truncated TFPI to inhibit TF/FVIIa
Strongly correlated	TFPI total antigen	Amount of full-length and truncated TFPI

TFPI, tissue factor pathway inhibitor; FXa, factor Xa; TF, tissue factor; FVIIa, factor VIIa.

long dPT. Correspondingly, low normalised TFPI anticoagulant ratios indicated low TFPI anticoagulant potential, whereas high ratios indicated high TFPI anticoagulant potential. Because of the limited amounts of plasma, most blood samples were assayed in a single run. Interday coefficient of variation (CV) was 2·8% and intraday CV was between 0·5% and 1·5%.

The 'TFPI free antigen and TFPI total antigen' were assayed in duplicate with commercial enzyme-linked immunosorbent assays (ELISAs) (Asserachrom® Free TFPI and Asserachrom® Total TFPI; Diagnostica Stago, Asniére, France), described in detail elsewhere (Dahm *et al*, 2003). Inter- and intra-assay variability, measured as CV was 4·4% and 2·9% for TFPI total antigen, and 4·9% and 3·8% for TFPI free antigen respectively. Bound TFPI was calculated as the difference between TFPI total antigen and TFPI-free antigen. The fraction of free, full-length TFPI in plasma was calculated as the proportion of TFPI-free antigen in TFPI total antigen.

The 'TFPI chromogenic substrate activity' was assayed in duplicate with an inhouse two-stage chromogenic substrate assay, as described earlier (Bendz *et al*, 2000). In this assay, TFPI activity was determined by the quantification of residual TF/FVIIa catalytic activity after the incubation of diluted plasma (containing TFPI) with TF, FVIIa in excess of TF-binding sites, and FXa. Inter- and intra-assay variability was 3·5% and 1·4% respectively.

Statistics

The controls represent the general population and were used to estimate the intravascular distribution of TFPI and to study the effect of hormonal state on dPT and the levels of TFPI.

To compare women using oral contraceptives (OC) with non-users, a special selection described earlier was made to obtain comparable groups (van Hylckama Vlieg *et al*, 2000). Non-menopausal women between 15 and 49 years of age were included. Women, who at the index date (similar date as date of thrombosis for patients) were pregnant, within 30 days postpartum, had a recent miscarriage or had used only depot contraceptives were excluded. A total of 142 control subjects were included in this analysis. To compare women not using OC with postmenopausal women, the same selection was used except that one postmenopausal woman who had used depot contraceptives was excluded.

To study the role of different forms of TFPI we calculated the risk for thrombosis associated with low TFPI for the different TFPI parameters. OC decrease TFPI levels in blood (Dahm *et al*, 2003). As OC is a well-known risk factor for DVT, many of the OC users with DVT stopped their use of OC at the time of diagnosis, and supposedly had higher TFPI at the time of blood sampling 6–56 months later. However, in the control group there was almost no change in OC use, hence OC users were omitted from the risk calculations (Dahm *et al*, 2003). Individuals on oral anticoagulation were excluded because of their effect on dPT, three patients with extreme TFPI anticoagulant values were excluded (Dahm *et al*, 2005). In addition, a few individuals had missing residual blood samples available for analysis. Therefore, this study involves 362 patients and 363 controls, which is less than in our previous report (Dahm *et al*, 2003). 'Low TFPI', for all TFPI parameters, was defined as TFPI below the 10th percentile among the controls as reported earlier (Dahm *et al*, 2003). In the calculation of the odds ratio (OR) for DVT in those who were low in both TFPI anticoagulant activity and TFPI chromogenic substrate activity, 'low TFPI' was defined as those individuals with TFPI below the 10th percentile in both assays at the same time, all others (i.e. those who had low TFPI in only one or none of the assays) were defined as having 'high TFPI'.

The TFPI values within groups of controls are reported as mean values and 95% confidence intervals (CI). Differences in mean values of normalised dPT between groups of controls with different hormonal state were tested with Student's *t*-test. *P* values below 0.05 were considered significant.

Results

Low TFPI anticoagulant activity as a risk factor for DVT

Normalised TFPI anticoagulant ratio below the 10th percentile gave an OR for DVT of 1·5 (95% CI 0·97–2·4) (Table II). To examine whether the TFPI anticoagulant activity assay assessed the risk for DVT differently from the other TFPI assays, ORs for DVT for TFPI levels below the 10th percentile were calculated in the same selection of cases and controls for TFPI free and total antigen, bound TFPI and TFPI chromogenic

Table II. Crude odds ratios for deep-vein thrombosis for normalised tissue factor pathway inhibitor (TFPI) anticoagulant ratio (n-TFPIac ratio), TFPI free antigen, TFPI total antigen, TFPI chromogenic substrate activity (TFPIcs activity) and bound TFPI.

	Patients ($n = 362$)	Controls ($n = 363$)	Odds ratio (95% CI)
n-TFPIac ratio			
>10th percentile	310	327	1·5 (0·97–2·4)
≤10th percentile	52	36	
TFPI-free antigen			
>10th percentile	316	327	1·3 (0·83–2·1)
≤10th percentile	46	36	
TFPI total antigen			
>10th percentile	314	326	1·3 (0·85–2·1)
≤10th percentile	48	37	
TFPIcs activity			
>10th percentile	317	324	1·2 (0·75–1·9)
≤10th percentile	45	39	
Bound TFPI			
>10th percentile	313	327	1·4 (0·90–2·2)
≤10th percentile	49	36	
n-TFPIac ratio and TFPIcs activity			
>10th percentile	345	360	5·9 (1·7–20)
≤10th percentile	17	3	

substrate activity. The ORs ranged from 1·2 to 1·4 (Table II), i.e. the risk for DVT was quite similar in all TFPI assays and none of the assays proved more predictive in assessing the risk for DVT. ORs in hormonal subgroups (men, postmenopausal women and premenopausal women not using OC) were in the same range for all assays (data not shown).

Those individuals who had low TFPI in both the activity assays, i.e. low TFPI chromogenic substrate activity and low normalised TFPI anticoagulant ratio, had an OR of 5·9 (1·7–20), relative to those who were only low in one or low in neither assay (Table II). Only three of the 363 (*c.* 1%) controls had low TFPI in both assays, in accordance with the expected prevalence based on the frequency of low values in each test of around 10%, which indicates that very few in the general population are affected by the risk for thrombosis due to low levels of TFPI in both activity assays. However, a separate study of the OC users in the healthy control group (who were excluded from other analyses, see Patients and methods) showed that 31 of 57 OC users (*c.* 54%) had low TFPI in both assays.

The effects of OC and hormonal state

Hormonal state had an explicit effect on the dPT. Premenopausal OC users had a shorter normalised dPT than non-users, and non-users had a shorter normalised dPT than postmenopausal women, who again had a shorter dPT than men. When TFPI was neutralised by anti-TFPI antibodies the difference in dPT between the hormonal subgroups were almost abolished, only some effect of male sex on dPT persisted (Table III), which means that the effect of hormonal state on dPT was mainly mediated through the effect of hormonal state on plasma levels of TFPI. Hormonal state had a corresponding effect on the normalised TFPI anticoagulant ratio. OC users had the lowest ratios and postmenopausal women and men had highest ratios (Table IV).

Estimation of the percentage-free TFPI of the total circulating TFPI in plasma

As the TFPI anticoagulant activity is a measure of the anticoagulant activity of the fraction of free full-length TFPI in plasma we investigated the effect of hormonal state on the fraction of free TFPI. The control group had a mean TFPI-free antigen/TFPI total antigen ratio of 0·19 (standard deviation 0·056, range 0·070–0·38). In other words, 19% of the total circulating TFPI was free full-length TFPI, but with large inter-individual variation mainly due to hormonal state. In OC users, approximately 13% was free full-length TFPI, while fertile women not using OC had a mean of 18% free full-length TFPI. Postmenopausal women and men had approximately 20% of their TFPI in the free form (Table V). Thus, it seems that hormonal state had a stronger effect on full-length than truncated TFPI in plasma.

Table III. Mean normalised diluted prothrombin time (dPT) with and without inhibiting anti-tissue factor pathway inhibitor (anti-TFPI) antibodies.

	OC+ ($n = 51$)	OC− ($n = 91$)	Postmenopausal women ($n = 83$)	Men ($n = 182$)
N-dPT	1·02**	1·12**	1·16**	1·20**
N-dPT + anti-TFPI	1·08 ns	1·10 ns	1·10 ns	1·13*

*$P = 0·01$; **$P < 0·01$; ns, non-significant. Comparisons are between adjacent categories; OC, oral contraceptives.

Discussion

We and other investigators have previously hypothesised that full-length TFPI plays an important biological role *in vivo* because of its strong inhibitory effect in dPT-based assays (Lindahl *et al*, 1991; Nordfang *et al*, 1991; Hansen *et al*, 1995; Sandset & Bendz, 1997). Hence, a dPT-based assay that can exclusively detect the functional activity of full-length TFPI might have the potential to detect TFPI deficiencies that other assays so far may have failed to detect (Sandset & Bendz, 1997; Ariens, 2005). Our current data do not support this hypothesis. TFPI anticoagulant activity below the 10th percentile was associated with a mildly increased risk for DVT, which was similar to the risks associated with levels of TFPI below the 10th percentile detected in conventional TFPI assays.

We have recently shown that the plasma levels of TFPI-free antigen and TFPI anticoagulant activity are closely associated, and that the levels of TFPI chromogenic substrate activity and TFPI total antigen also are closely associated (Table I) (Dahm *et al*, 2005). Therefore, it hardly seems necessary to use both the antigen assays and the activity assays in population-based studies. Measurements of TFPI free and total antigen will give a good impression of the TFPI anticoagulant activity and the TFPI chromogenic substrate activity, respectively, as well as of the risks for DVT associated with each assay. But in individuals with a suspected mutation, dual antigen and activity assay would be of interest to detect a dysfunctional protein.

The TFPI anticoagulant activity and TFPI chromogenic substrate activity are not very closely associated and do probably reflect different targets of inhibition *in vivo* (Dahm *et al*, 2005). Based on this and other reports on the kinetics of the inhibition of FXa and TF/FVIIa by TFPI (Huang *et al*, 1993; Lindhout *et al*, 1994, 1995; Lockett & Mast, 2002), we suggest that the TFPI anticoagulant activity is a measure of the inhibition of FXa, while the TFPI chromogenic substrate activity is a measure of the inhibition of the TFPI/FXa complex (Table I). TFPI anticoagulant activity and TFPI chromogenic substrate activity may therefore be considered to be independent risk factors for thrombosis. Moreover, both activity assays yielded approximately the same risk for DVT even though they were not closely associated [Table I and (Dahm *et al*, 2005)]. We have tentatively calculated the risk for DVT for the

Table IV. Mean normalised tissue factor pathway inhibitor anticoagulant (n-TFPIac) ratio (95% CIs) in controls.

	OC users ($n = 51$)	OC non-users ($n = 91$)	Postmenopausal ($n = 83$)	Men ($n = 182$)	All ($n = 436$)
n-TFPIac ratio	0·95 (0·94–0·95)	1·01 (1·00–1·02)	1·05 (1·04–1·07)	1·07 (1·06–1·07)	1·03 (1·03–1·04)

OC, oral contraceptives.

Table V. Mean proportion of tissue factor pathway inhibitor (TFPI)-free antigen (95% CI of mean) in plasma in controls.

	OC users ($n = 54$)	OC non-users ($n = 99$)	Postmenopausal ($n = 89$)	Men ($n = 201$)	All ($n = 473$)
Proportion TFPI-free antigen (%)	12·5 (11·5–13·5)	17·5 (16·5–18·4)	19·7 (18·6–20·8)	20·6 (19·9–20·9)	18·7 (18·2–19·2)

OC, oral contraceptives.

individuals who had both low TFPI anticoagulant activity and low chromogenic substrate activity below the 10th percentile, and found that low levels were associated with an almost sixfold increase in the risk for DVT. The CI demonstrates the uncertainty of this risk estimate, and the usefulness of the combined measures warrants confirmation in other studies.

We also find it interesting that OC use was associated with the combination of low levels of TFPI anticoagulant activity and TFPI chromogenic substrate activity in >50% of users, when compared with <1% in the control subjects not using OC. These data further substantiate the potential important role that TFPI may play in increasing the risk of venous thrombosis in OC users.

Hormonal state had an effect on the normalised TFPI anticoagulant ratio comparable with that shown previously (Harris *et al*, 1999; Hoibraaten *et al*, 2001; Dahm *et al*, 2003). Hormonal state, especially OC use, also affected the normalised dPT by shortening the clotting times. However, addition of anti-TFPI antibodies that completely ameliorated the effects of TFPI, resulted in nearly identical clotting times independent of hormonal state. Hence, the effects of OC and hormonal state on TF-initiated clotting time can, to a large extent, be attributed to the effects on full-length TFPI. This is supported by our recent finding that there was a clear relationship between the normalised dPT and plasma level of TFPI-free antigen, but not bound TFPI and anti-TFPI antibodies repressed this effect (Dahm *et al*, 2005). This result also indicates that all assays triggered with TF, e.g. endogenous thrombin potential-based activated protein C resistance (de Visser *et al*, 2005), are possibly affected by the level of full-length TFPI in the sample assayed.

The fractions of free TFPI in plasma have been estimated by several authors using different methods (Novotny *et al*, 1989; Lindahl *et al*, 1991; Hansen *et al*, 1994), and the results have varied from 3% to 20%. In the present study, the fraction of free full-length TFPI was approximately 20% using two different ELISAs with detecting monoclonal antibodies directed against either the first 160 amino acid residues of the TFPI molecule in the TFPI total antigen assay or against the last 161–240 residues in the TFPI-free antigen assay (Dahm *et al*, 2003). We have recently confirmed *in vivo* (Dahm *et al*, 2005) that the full-length TFPI identified by the TFPI-free antigen assay had the same ability to inhibit coagulation as the full-length TFPI investigated *in vitro* (Lindahl *et al*, 1991, 1992; Hansen *et al*, 1994, 1997). Hence, we believe that we have made a fairly good estimate of the fraction full-length TFPI circulating in the blood. Hormonal state, especially OC use influenced the fraction of free TFPI profoundly, which suggests that hormonal state affects full-length TFPI in plasma more than truncated TFPI.

The assumption that full-length TFPI is biologically more important than truncated forms has been based on *in vitro* studies, and our study is the first to test this hypothesis *in vivo*. Our data suggest that full-length TFPI is not more important than truncated TFPI *in vivo*, but that both forms play a role in the protection against thrombosis. The increased risk related to the combined measure of the two activity assays suggest that the ability of TFPI to inhibit either FXa or TF/FVIIa, or both, still might be important for its anticoagulant effects *in vivo*. A more fundamental question is whether circulating TFPI actually plays any significant role *in vivo*. It could very well be that the most important pool of TFPI *in vivo* is the much larger pool of TFPI bound to the endothelium. The ORs observed for different plasma forms of TFPI could possibly be faint, and inaccurate, reflections of risks connected to the endothelium-associated TFPI, but this needs investigation in other studies.

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