**Introduction**

Tissue factor pathway inhibitor (TFPI) is a potent anticoagulant protein comprised of three Kunitz-type domains flanked by less-structured peptide segments. TFPI’s anticoagulant function has been well characterized and involves two basic steps of inhibition. The first step involves the binding of TFPI to factor Xa (FXa), while in the second step, the TFPI-FXa complex binds and inhibits the TF-factor VIIa (FVIIa) complex, thereby forming a quaternary complex. These inhibitory mechanisms involve two Kunitz domains of TFPI. Kunitz domain 1 inhibits the FVIIa complexed to TF, while Kunitz domain 2 inhibits FXa. No direct protease-inhibiting functions have been demonstrated for the K3 domain. Importantly, the FXa-TFPI complex is a much more potent inhibitor of the FVIIa-TF complex than is TFPI by itself (1).

The role of TFPI in thromboembolic diseases, however, is only poorly understood. To date, no patients with TFPI deficiency have been reported, perhaps because homozygous TFPI deficiency is likely to be a lethal condition. This is supported by the...
observation that TFPI knockout mice die early in the embryonic state due to massive consumptive coagulopathy (2). Another reason that TFPI deficiency is difficult to detect is that only a small fraction (about 10 to 15%) of TFPI is present in the circulating plasma, while the major part of TFPI is bound to the endothelium (3). Thus, the plasma levels of TFPI do not reflect the total-body levels of TFPI. As shown recently, the biggest part of the endothelial TFPI pool seems to be associated with endothelial GPI-anchored proteins whereas a minor fraction can be mobilized by heparin (4, 5). TFPI deficiency has also been detected by measuring TFPI levels after the administration of heparin. In a small study post-heparin TFPI levels were found more often decreased in patients with arterial or venous thrombosis than in the controls (6). Further studies, however, are needed to confirm these preliminary observations. In patients with disseminated intravascular coagulation (DIC) due to sepsis or malignancy, TFPI deficiency has also not been found consistently. In contrast, TFPI levels have more often been shown to be significantly elevated, which probably reflects endothelial damage (7–9). Additionally, three polymorphism sites in the TFPI gene have been reported, including the V264M mutation (10), the C399T mutation (11), and the T287C mutation (12). Although these polymorphisms have been found in the normal population and in patients with arterial or venous thrombosis, there is so far no evidence that these mutations are associated with either arterial or venous thrombosis and are therefore not relevant risk factors.

Due to this lack of evidence for the clinical relevance of TFPI deficiency, based on either TFPI protein levels or mutations in the corresponding gene, we hypothesized that in patients with thromboembolism TFPI would be present at normal levels as a fully active protein but would be limited in its anticoagulation activity due to alterations of its substrates including FVII, FX, or TF. In other words, we suggested the presence of a TFPI resistance that is analogous to activated protein C (APC) resistance. To test this hypothesis, we developed a TF-based clotting assay in which the anticoagulant response to purified TFPI was measured. To determine whether a reduced anticoagulant response to TFPI would be associated with a thrombotic risk, we compared 118 healthy controls to 120 patients with documented venous thromboembolism for whom hereditary and acquired thrombophilia had been excluded.

**Materials and methods**

**Controls and patients**

From the beginning of 1999 until July 2002, patients who had been admitted to our department for thrombophilia screening were consecutively included in the study if they had a history of one or more venous thromboembolic events (ie, deep-vein thromboses (DVTs) of the lower extremities with or without pulmonary embolism or isolated pulmonary embolism). Patients were included only if the thromboembolic event was documented by imaging techniques including venography, ultrasonography, ventilation-perfusion scanning, spiral computerized tomography, or pulmonary angiography. Further inclusion criteria were the absence of acquired or hereditary thrombophilia including that caused by lupus anticoagulant, anti-cardiolipin-immunglobulin G (IgG/IgM, anti-β2-glycoprotein-IgG/IgM, FV Leiden mutation, prothrombin gene mutation G20210A, or deficiencies of protein C, protein S, or antithrombin.

The exclusion criteria were the following: elevated levels of C-reactive protein (CRP), a time interval of less than 3 months between laboratory testing and the thromboembolic event, the presence of complications likely to be associated with high TFPI levels such as malignancy and inflammatory diseases, and patients undergoing oral anticoagulation therapy.

Serving as the control group, 118 healthy volunteers (medical students and hospital staff) without histories of venous or arterial thrombosis and not taking any medications were examined consecutively within the same period of time and at a similar collection rate as the experimental group. In all controls the presence of the thrombophilia and the additional exclusion criteria as mentioned above has also been excluded. The clinical and demographic data of the patients and controls are summarized in Table 1.

**Thrombophilia tests**

Blood was collected by venipuncture in tubes containing 0.106 mmol of trisodium citrate per liter. Plasma was prepared by centrifugation for 10 minutes at 2,000 × g at room temperature. Functional antithrombin, protein C, and protein S were measured with Coamatic Antithrombin (Chromogenix, Mölndal, Sweden), Berichrom Protein C (Dade-Behring, Marburg, Germany), and Staclot Protein S (Diagnostica Stago, Asnière, France), respectively. APC resistance was assessed by the APC-dependent prolongation of the activated partial thromboplastin time (aPTT) in citrated plasma according to the manufacturer’s instructions (Coatest APC Resistance, Chromogenix). Lupus anticoagulant was determined by a Russel’s viper venom test (LA1 screening and LA2 confirmation test, Dade Behring). Genetic analyses of the FV Leiden mutation R506Q and prothrombin gene mutation G20210A were performed with the LightCycler FV Leiden and prothrombin mutation detection kits (Roche Diagnostics, Rotthrist, Switzerland). Anti-cardiolipin and anti-β2-glycoprotein-1 antibodies were measured by enzyme-linked immunosorbent assay (ELISA) kits (Coaliza Anti-cardiolipin, Chromogenix and Varelisa β2-GP-1, Elias, Freiburg, Germany).

**TFPI resistance test**

Blood was collected by venipuncture in tubes containing 0.106 mmol of trisodium citrate per liter, and plasma was pre-
pared by centrifugation for 10 min at 2,000 × g at room temperature. Plasma samples were then immediately frozen and stored at –70°C. After the collection period of 3.5 years, all samples have then been thawed and analyzed together at the same time. When compared with freshly obtained plasma samples, such long term storage did not influence the assay results. To prepare a TF-dependent clotting-inducing solution, a prothrombin time reagent (Thromborel S®, Dade-Behring) was diluted 1:30 in Tris (tris(hydroxymethyl)aminomethane)-HCl buffer (50 mM Tris-HCl, 175 mM NaCl, 5 mM CaCl₂, 0.5 mg/mL of bovine serum albumin (BSA), pH 7.9). The rational for the 1:30 dilution of the prothrombin reagent was to obtain only a moderate induction of coagulation which was sensitive enough to the inhibitory effect of exogenously added TFPI. Dilutions of more than 1:30 were, however, not appropriate because of the high imprecision of the long clotting times. This solution was named solution A. A second solution (named solution B) was prepared using the same ingredients as solution A but with the addition of functional, recombinant TFPI (human TFPI activity standard, American Diagnostica GmbH, Pfungstadt, Germany) at a final concentration of 0.3 µg/mL (8.6 nM). TFPI was added in pure form without the addition of any carriers. For the clotting assay, the test plasma was mixed with FVII-deficient plasma at a ratio of 2 to 3 and incubated for 1 minute at 37°C. The rational for this mixing was to equalize individually different levels of coagulation factors (except FVII) and, thus, to minimize any possible influences of other coagulation factors on the assay. The best mixing ratio with regard to assay precision was found to be 2 parts of test plasma and 3 parts of FVII-deficient plasma. After putting 50 µl of this mixed test plasma into a clotting analyzer (STA 4, Diagnostica Stago), clotting was started by adding either 100 µl of solution A or 100 µl of solution B. The result of the clotting time with solutions A (without TFPI) and B (with TFPI) was expressed as the ratio of B to A. For each run with test samples, a control was performed by using normal human pool plasma. Control experiments using this method revealed an excellent reproducibility showing a constant TFPI response upon time in the same plasma sample of the same individual.

Influence of individual coagulation factors on the TFPI resistance test
Since it has been shown that FVIII and FIX can individually reduce the ability of TFPI to inhibit the TF-FVIIa complex resulting in a 20-fold-increased FXa formation (12, 13), we further performed a series of experiments where the influence of various coagulation factors on the TFPI sensitivity ratio was tested. In 50 healthy individuals we measured the TFPI sensitivity ratio by using an assay in which the test plasma was not mixed with FVII-deficient plasma. The results of these TFPI sensitivity ratios were then correlated with the individual levels of fibrinogen, FII, FV, FVII, FVIII, FIX, and FX. Using an automated coagulometer (Behring Coagultion System, Dade-Behring, Marburg, Germany) fibrinogen was determined with the method of Clauss and all other coagulation factors were measured by commercial clotting rate assays (Dade-Behring).

Table 1: Summary of the study population

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Patients</th>
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<tbody>
<tr>
<td>Individuals, n</td>
<td>118</td>
<td>120</td>
</tr>
<tr>
<td>Mean age (± SD)</td>
<td>39.6 (± 11.1)</td>
<td>46.3 (± 12.4)</td>
</tr>
<tr>
<td>Male sex (% of patients)</td>
<td>40 (33.9 %)</td>
<td>44 (36.6 %)</td>
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<tr>
<td>Thromboembolic events:</td>
<td></td>
<td></td>
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<tr>
<td>– isolated deep vein thrombosis</td>
<td>-</td>
<td>68 (56.7 %)</td>
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<tr>
<td>– isolated pulmonary embolism</td>
<td>-</td>
<td>19 (15.8 %)</td>
</tr>
<tr>
<td>– deep vein thrombosis and pulmonary embolism</td>
<td>-</td>
<td>33 (27.5 %)</td>
</tr>
<tr>
<td>Circumstantial risk factors (immobilisation, postoperative, oral contraceptives, hormone replacement therapy, pregnancy, long-distance flight, plaster cast)</td>
<td>-</td>
<td>91 (75.8 %)</td>
</tr>
<tr>
<td>Idiopathic thromboembolism (no circumstantial risk factors)</td>
<td>-</td>
<td>29 (24.2 %)</td>
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Statistical analysis
The statistical significance of the difference of the TFPI sensitivity ratios between the healthy controls and the patients was calculated by the Mann-Whitney test. Confidence intervals (CIs) of 95% were used. Relations between TFPI sensitivity ratios and plasma levels of coagulation factors were analyzed by correlation coefficients. A multivariate analysis was performed using stepwise regression. To address the problem of multiple comparisons, correlations were considered statistically significant only for p-values less or equal 0.025 (Bonferroni correction).
Results

Performance of the TFPI resistance test
As shown in Figure 1, the addition of recombinant TFPI to normal human pool plasma (diluted with FVII-deficient plasma) at increasing concentrations produced a progressive prolongation of the diluted prothrombin clotting time. At a final concentration of TFPI of 1.5 µg/mL, the clotting time started to become indefinitely prolonged (>500 seconds). At concentrations between 0.2 and 0.5 µg/mL, the clotting times were highly consistent and reproducible (160 to 220 s), so a final concentration of 0.3 µg of TFPI/mL was chosen to determine the TFPI sensitivity ratio of the patients and controls. Using this TFPI concentration together with the assay specifications as described above, the intra-assay and inter-assay coefficient of variation was 2.3 % (n= 10, mean 1.69, SD 0.039) and 4.6 % (n = 10, mean 1.66, SD 0.076), respectively.

Since the anticoagulant effect of TFPI is known to be increased in the presence of heparin (15) or after complex formation with purified FXa (16), we evaluated several assay modifications using either TFPI with heparin or TFPI complexed with FXa. The addition of heparin, however, produced very inconsistent results with often uncontrollable prolongation of the clotting times. The preincubation of TFPI with purified FXa was also cumbersome and problematic, sometimes leading to an even further decreased anticoagulant effect, as has also been demonstrated by other investigators (17). All in all, this modification did not reveal a clear advantage.

Since it was conceivable that one or more coagulation factors could influence the inhibitory effect of TFPI in our assay, we determined the levels of fibrinogen, FII, FV, FVII, FVIII, FIX and FX in 50 healthy individuals. To better see an influence of one of these clotting factors on the activity of TFPI, we measured the TFPI sensitivity ratio without mixing the test plasma with FVII-deficient plasma. As shown in Table 2, none of these seven clotting factors, however, correlated significantly with the TFPI sensitivity ratio. We found only a slight, insignificant correlation between the levels of FVII and FX with the TFPI sensitivity ratio. In particular, there was a tendency for high levels of FVII or FX to more often be associated with a low ratio.

TFPI sensitivity ratio in patients and controls
When tested in the 118 healthy controls and 120 patients, the TFPI sensitivity ratio produced values between 1.25 and 2.0 in both groups. The mean values, however, were significantly lower (P < 0.001) in the patient group (1.49 ± 0.15 SD) compared to the controls (1.62 ± 0.16 SD). If the normal range is considered to be between 1.31 and 1.93 (mean value ± 2 SD, 1.62 ± 0.31) based on the values of the 118 controls, 15% of the patients and 3.4% of the controls had ratios that, by these criteria, are considered pathological.

Discussion

TFPI is an important natural anticoagulant. This has repeatedly been demonstrated by the successful use of recombinant TFPI as an antithrombotic agent in different thrombosis models and thromboembolic diseases (1). Despite this potent effectiveness, however, convincing evidence of relevant quantitative or qualitative TFPI deficiencies has not been reported so far, nor could...
three different TFPI polymorphisms be consistently associated with arterial or venous thromboembolism (10–12). Thus, we thought that not the TFPI protein itself but rather its substrates, including FVII, FX, or TF, could be the reason for a pathological function leading to a hypercoagulable state, analogous to the mechanism of APC resistance.

We therefore designed a TFPI sensitivity clotting assay in which the anticoagulant response to exogenous recombinant TFPI was analyzed. Before using the assay in a large population of controls or patients, numerous modifications were tested to obtain the best possible precision, accuracy, and stability. A main goal was to establish conditions under which the difference between the clotting time with and without TFPI was as large as possible so that a ratio high enough to differentiate between good and poor responses was achieved. Another main goal was to establish conditions under which high or low individual levels of other indirectly involved clotting factors could not hamper the anticoagulant effect of TFPI. Although we did not find any significant correlation between the level of a coagulation factor with the TFPI sensitivity ratio, we decided nonetheless to mix the test plasma with FVII-deficient plasma (at a ratio of 2:3) to avoid any influences of other clotting factors. It was clear that any potential defect in FX, the other substrate of TFPI, leading to a reduced response to TFPI, could possibly be...
missed. However, after comparing all samples with both assays, one using FVII-deficient plasma and one without using additional plasma, we did not find any differences besides a better reproducibility of the assay with FVII-deficient plasma.

To test whether TFPI resistance would indeed be a novel thrombophilia factor that could explain the cause of thromboembolism in patients with so far unexplained venous thrombosis, we decided to evaluate this assay only in patients for whom other common thrombophilic states had been excluded. Thus, none of the 120 patients tested positive for APC resistance, prothrombin gene mutation G20210A, antithrombin or protein C/protein S deficiency, or antiphospholipid antibodies. As shown in Figure 2, the patients had significantly lower TFPI sensitivity ratios than the controls. Since it is so far unknown whether a decreased response to TFPI is due to a genetically (and probably also functionally) altered FVII or FX, it is therefore not possible to determine an exact cutoff value that would allow the safe discrimination between pathologic and normal TFPI sensitivity ratios. However, it is conceivable that, independent of a specific mutation in one of the substrates of TFPI, a decreased response to TFPI could still be associated with an increased risk of thrombosis, as has been shown in patients with APC resistance (18). A common method for determining risk estimations is to compare the prevalences of patients whose values fall below the 10th percentile of the controls with those falling above the 90th percentile of the controls. Of the 120 patients, 39 (32.5%) had a TFPI sensitivity ratio below the 10th percentile of the controls, compared with 11 (9.3%) of the healthy controls. Only 3 patients (2.5%) were above the 90th percentile of the controls, while there were 11 (9.3%) persons among the healthy controls. Hence, the crude odds ratio for venous thrombosis for subjects with a TFPI sensitivity ratio below the 10th percentile was 13 (95% CI; range, 3.1 to 54.9) compared with those with a ratio above 1.8 (90th percentile). In accordance with these results, a recently published study using a very similar assay to test the individual response to exogenously added TFPI has also shown a significantly higher prevalence of TFPI resistance among patients with thromboembolism than in healthy controls (19). Using a somewhat different cutoff calculation these authors found a poor anticoagulant response to TFPI in 4.7% of the controls and 11.0% in the patients.

Besides the absence of a major hereditary or acquired thrombophilia, about 25% of our patients had a thromboembolic event without a circumstantial risk factor, thus offering no explanation for a prothrombotic state. It was therefore of particular interest to check whether these patients had especially low TFPI sensitivity ratios. As shown in Figure 2, however, the TFPI sensitivity ratios of patients with idiopathic thrombosis were distributed over the entire reference range as with the other thrombosis patients. Likewise, the study from Tardy-Poncet et al. also revealed no accumulation of patients with idiopathic thrombosis among those patients with a particular low anticoagulant response to TFPI (19). In addition, it was of interest to see whether the TFPI sensitivity ratios differed among patients with either isolated deep vein thrombosis or isolated pulmonary embolism as it has been demonstrated in patients with FV Leiden mutation and elevated FVIII levels (20, 21). We were, however, not able to detect a difference in the distribution of the TFPI sensitivity ratios between these two groups.

In conclusion, we found that in the plasma of patients with a history of venous thromboembolism and otherwise no evidence for hereditary or acquired thrombophilia, the anticoagulant response to recombinant TFPI was significantly reduced compared to the anticoagulant response in healthy controls. Whether such a TFPI resistance is based on an altered FX, FVII, or another unknown mechanism altogether remains to be further elucidated.

References


21. Bombeli T, de Conno E, Jutzi M, et al.. In patients symptomatic for deep-vein thrombosis factor VIII elevation is found twice as frequent as in patients symptomatic for pulmonary embolism. Thromb Haemost 2003; 89: 198-200