Soluble thrombomodulin activity and soluble thrombomodulin antigen in plasma

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Summary. Endothelial cell membrane-bound thrombomodulin (TM) plays a critical role as a cofactor in the protein C pathway, important in regulating coagulation as well as inflammation. Heterogeneous soluble TM fragments circulate in the plasma and are found at increased levels in various diseases such as cardiovascular disease and diabetes, and in ischemic and/or inflammatory endothelial injuries. The anticoagulant function of these soluble fragments has not been measured in healthy individuals or in patients. Using an immobilized monoclonal antibody against TM and a microtiter plate format, an assay was designed to capture the soluble TM fragments in plasma and measure their cofactor activity in the thrombin-mediated activation of protein C. In addition, soluble TM antigen levels were measured by enzyme-linked immunosorbent assay. Both assays were used to investigate a group of healthy blood donors. TM fragments released into plasma were shown to retain significant cofactor activity, and reference intervals for healthy men and women were established. Furthermore, a statistically significant correlation was observed between soluble TM antigen levels and soluble TM cofactor activity. This notwithstanding, soluble TM activity only accounted for a minor part of all variation in soluble TM antigen levels (R^2 = 22% in men and R^2 = 16% in women).

Keywords: anticoagulant activity, protein C pathway, thrombomodulin.

Introduction

Activation of protein C is of central importance in regulating not only the coagulation process but also the inflammatory reaction [1]. Once activated, protein C is a potent anticoagulant and anti-inflammatory protease.

Thrombomodulin (TM) is an endothelial cell membrane protein that plays an important role in the protein C system. This protein binds to thrombin and converts it from a procoagulant protease to an anticoagulant enzyme that activates protein C. This establishes the anticoagulant and anti-inflammatory functions of TM [2]. In addition, the TM–thrombin complex activates thrombin-activatable fibrinolysis inhibitor (TAFI), which also confers antifibrinolytic properties to TM [3,4]. Hypothetically, an impaired TM function might sway the inflammatory balance in situations such as a sepsis, thereby adding another clinically important role for this endothelial transmembrane protein.

Heterogeneous soluble TM fragments (sP-TM antigen) circulate in plasma and urine [5], as well as in synovial fluid [6]. Levels of the sP-TM antigen are raised in various clinical conditions such as cardiovascular disease and diabetes, and in ischemic and/or inflammatory endothelial injuries. Concerning the functional activity of the sP-TM antigen, it has been demonstrated that the TM fragments in plasma inhibit fibrinolysis through the activation of TAFI [7]. However, it is not clear whether they also function in plasma as a physiologically significant anticoagulant in the thrombin-mediated activation of protein C. Two research groups have measured the cofactor activity of TM molecular subspecies isolated from plasma in the thrombin-mediated activation of protein C, and reported that the activity was 30–50% compared to that of cellular TM [5,8]. Isolated human urinary soluble TM also accelerates protein C activation in human plasma [9].

Separation and characterization of plasma TM fragments have resulted in reports of four [10], six [11], or seven [8] molecular subspecies. A difference in the proportions of the molecular subspecies between diabetic patients and healthy controls was demonstrated [8].

We have designed an assay to measure plasma TM activity (sP-TM activity). Using this assay, we have studied the TM cofactor activity in the thrombin-mediated activation of protein C in plasma from healthy blood donors and report a reference interval and a comparison between soluble TM
activity and circulating levels of soluble TM fragments or antigen in plasma.

Materials and methods

Materials

Soluble TM antigen levels in plasma were measured with a TM enzyme-linked immunosorbent assay (ELISA) as reported earlier [12]. A monoclonal antibody (mAb) against human TM, mAb TM43b [13] and a recombinant truncated and soluble form of TM, Solulin [14], were kind gifts from Berlex Biosciences in San Francisco, CA. A polyclonal antibody against TM was also obtained from Berlex. The TM-unrelated polyclonal antibody was directed against the human plasma protein Cystatin C, and was a kind gift from Prof. Anders Grubb in our laboratory. Thrombin [15,16], and protein C [17] used in the assay were isolated in our laboratory as described in the literature.

Blood samples

Fifty men (mean age 38 years, range 18–65) and 50 women (mean age 37 years, range 20–50) were recruited from the local blood clinic. The local Human Subject’s committee gave approval for the study. The donors were informed that their blood was being drawn for research purposes and that their identity would not be revealed. Blood samples were obtained in EDTA and 3.8% citrated blood was being drawn for research purposes and that their blood was being drawn for research purposes and that their identity would not be revealed. Blood samples were obtained in EDTA and 3.8% citrated

Measuring TM activity in normal human plasma

Soluble TM activity in plasma was studied with a newly designed assay. A mAb against human TM, mAb TM43b [13], was coupled to a microtiter plate (Nunc Microwell Plate Flat Well Polypropylene) according to standard procedures (100 µL, 10 µg mL⁻¹ mAb TM43b per well) described by the manufacturer (Nunc A/S, Roskilde, Denmark).

A recombinant truncated and soluble form of TM, Solulin, was used as a standard [14]. In this assay, we have defined the antigen level or activity of 1 ng Solulin as 1 Solulin Equivalent (SEQ). A stock solution of 500 ng mL⁻¹ Solulin (concentration determined by acid hydrolysis and amino acid analysis) was diluted to 5, 2.5, 1.25, 0.63, 0.32, 0.16, and 0.08 ng mL⁻¹ with phosphate-buffered saline (PBS; 10 mmol L⁻¹ sodium phosphate, 0.15 mol L⁻¹ NaCl, pH 7.4) containing 0.1% dry milk and 0.1% Tween. Before the assay, the plasma samples were diluted 1 : 4 in the same buffer.

In the first incubation, 200 µL of blank (PBS with 0.1% dry milk and 0.1% Tween-20), standard or diluted plasma was added to a TM43b-coated microtiter plate in duplicate. The plate was covered with adhesive tape and incubated for 2 h at room temperature under careful shaking using an IKA-VIBRAX-VXR to bind Solulin or TM fragments. All fluid was removed from the wells, and the plate was washed three times with 300 µL PBS containing 0.1% dry milk and 0.1% Tween. In the second incubation, 50 µL 8.8 mmol L⁻¹ thrombin (in 20 mmol L⁻¹ HEPES, 0.15 mol L⁻¹ NaCl, 2 mmol L⁻¹ CaCl₂, 0.1% Tween, pH 7.4) and 50 µL 0.4 µmol L⁻¹ human protein C (in 20 mmol L⁻¹ HEPES, 0.15 mol L⁻¹ NaCl, 2 mmol L⁻¹ CaCl₂, 0.1% Tween, pH 7.4) were added to the wells. The plate was again covered with adhesive tape and incubated for 3 h at 37 ºC under slow shaking.

Following this incubation and protein C activation, 10 µL Hirudin (Refludan®) at 132 IU mL⁻¹ (in 20 mmol L⁻¹ HEPES, 0.15 mol L⁻¹ NaCl, 2 mmol L⁻¹ CaCl₂, 0.1% Tween, pH 7.4) was added to each well to inhibit thrombin activity. The plate was incubated at 37 ºC for 10 min while the plate reader (Spectramax 340, Molecular Devices, Göteborgs termometerfabrik, Göteborg, Sweden) was prepared. Finally, 75 µL of 2 mmol L⁻¹ S-2366 (Chromogenix, Göteborg, Sweden) in 20 mmol L⁻¹ HEPES, 0.15 mol L⁻¹ NaCl, 2 mmol L⁻¹ CaCl₂, 0.1% Tween, pH 7.4 was added to each well and the absorbance increase at 405 nm was monitored for 30 min. The activity that was measured in the blank sample was automatically subtracted from all standard and patient values.

Determination of the TM specificity of the assay

To confirm that the measured activity was TM-specific, the following experiments were performed in duplicate. First, before carrying out the assay, an approximately 1000-fold molar excess of an anti-TM polyclonal antibody or an unrelated polyclonal antibody (anti-human Cystatin C) was added to the standards and three normal plasma samples. The sP-TM activity for each sample was measured using the described assay.

Recovery of TM activity added to plasma

Solulin was added to normal pooled EDTA and citrated plasma (0.08, 0.16, 0.32, 0.64, 1.25, 2.5 and 5 ng mL⁻¹ final concentrations). The sP-TM activity for each sample was measured using the described assay.

Statistical analyses

Ordinary linear regression was applied with sP-TM activity as a dependent variable and sP-TM antigen level as an independent variable. The regression modeling was conducted for men and women separately. The non-parametric Friedman test was used
in the comparison of the obtained values for the plasma samples from blood donors at the three different time-points. When comparing pairwise differences from baseline to either 3 months or 1 year values with percentage limits, Wilcoxon Signed Rank test was used. Differences yielding $P < 0.05$ were regarded as statistically significant.

**Results**

**Soluble TM activity in plasma**

The soluble TM fragments circulating in plasma were captured in microtiter wells using an anti-TM mAb TM43b that does not prevent thrombin binding or protein C activation by the thrombin–TM complex [13]. The cofactor activity of the isolated TM fragments in the thrombin-mediated activation of protein C was measured using as standard a truncated recombinant TM variant, Solulin.

During assay development most variables were studied separately (not shown), except for the amount of the mAb used in the wells in the first step where we followed the manufacturer’s instructions. For each variable, the aim was to define the volume or concentration resulting in the highest protein C activation rate consistent with low blank value and high precision. Sample volumes ranging from 100 to 200 µL were tested for both standards and normal blood samples. $V_{max}$ was found to increase with increasing volume, but for practical reasons volumes above 200 µL (the fixed volume) could not be used.

Washing volumes (200–300 µL) and number of repetitions (three to five times) were investigated and optimized to $3 \times 300$ µL. Plasma samples were run undiluted and diluted from 1:2 to 1:8. Dilution from 1:4 to 1:8 gave equivalent results, but dilution higher than 1:4 (the fixed value) resulted in unacceptable imprecision as a result of the difference between blank and sample being too low. In the activation step, calcium ion concentration (0–6.0 mmol L$^{-1}$), thrombin concentration (0.5–32 mmol L$^{-1}$) and protein C concentration (0.1–0.4 µmol L$^{-1}$) was tested. The fixed values for the final concentrations of these reagents in the activation step were 2 mmol L$^{-1}$, 4 mmol L$^{-1}$ and 0.2 µmol L$^{-1}$, respectively; they were chosen to give maximal $V_{max}$ and the lowest imprecision coupled with reasonable consumption of materials. We also characterized the rate of generation of APC in our assay by measuring the amount of APC formed at different time-points from the addition of thrombin and protein C to the wells. Under the assay conditions that we use the activation rate of protein C was always linear for at least 20 h. For 50 of the blood donor samples, we also compared the results when performing the activation step for 3 h and 20 h. There was no significant difference. Therefore, we decided to monitor the activation step as an end-point measurement of the amount of APC that was formed after 3 h of protein C activation.

Finally, various blocker compositions in the buffers were tested, including bovine serum albumin, dry milk, and/or detergents. The dry milk + detergent resulted in the highest $V_{max}$ in the second step.

The cofactor activity of TM in individual samples was calculated by comparing the mean $V_{max}$ from the duplicate wells to the standard curve. TM activity as well as TM antigen level was expressed as SEq mL$^{-1}$, and the quotient sTM activity/sTM antigen was also calculated for each sample. The standard curve was always linear from 0 to 5 ng mL$^{-1}$ (not shown). The intra-assay and the interassay coefficients of variation were <8% and <15%, respectively. The detection limit of the assay was 0.03 SEq mL$^{-1}$ (+3SD for the blank sample).

**TM specificity of the assay**

We wanted to confirm that the measured activity was TM-specific. To that end, we pre-incubated all standards and three normal plasma samples with an approximately 1000-fold molar excess of an anti-TM polyclonal antibody or an unrelated polyclonal antibody before carrying out the assay. In all wells where the standard or plasma sample was pre-incubated with an anti-TM antibody, the assay result was comparable to the blank value. In contrast, an unrelated polyclonal antibody did not affect the TM activity of the standards or the normal plasmas (results not shown).

Second, we also performed the assay according to the protocol for a blank sample and a standard sample, Solulin 5 ng mL$^{-1}$, but replacing thrombin or protein C with buffer. When protein C was excluded, there was no detectable activity in any of the samples (the thrombin was effectively inhibited by Hirudin both in its free form and in complex with TM/Solulin), but with thrombin excluded we could always measure a minor activity and in both the blank and the Solulin sample. This minor activity varies between different protein C preparations and we consider this slight activity a result of a tiny amount of activated protein C already existing in the protein C preparation. We always measure the same varying, but tiny, activity in the blank sample when protein C as well as thrombin is present and we always correct the measured value from each standard point and each patient sample by subtracting the activity against S-2366 in the blank sample.

**Recovery of TM activity added to plasma**

To ensure that the TM activity assay measures the expected activity of TM supplemented into normal plasma with its complement of endogenously derived TM fragments, various concentrations of Solulin (0.08–5 ng mL$^{-1}$) were added to normal pooled EDTA and citrated plasma. The normal pooled plasmas gave a P-TM activity of 3.4 SEq (EDTA) or 3.5 (citrate) when no Solulin was added.

Figure 1 shows the measured TM activity plotted against the Solulin concentrations that were supplemented into normal pooled plasma. The slopes of the lines were 0.94 (EDTA), 1.05 (citrate) and the $R^2$ values were 0.99 and 1.0, respectively.
SP-TM activity and sP-TM antigen and sample storage

Plasma samples from healthy blood donors stored at −70 °C and thawed only once were analyzed at baseline and at 3 and 12 months. The measurements gave significant differences for sP-TM activity (EDTA and citrate, \( P < 0.01 \)), as well as for sP-TM antigen (EDTA, \( P < 0.001 \) and citrate, \( P = 0.015 \)). No time trends could be detected. In contrast, there seemed to be a random variation from time-point to time-point. The variation from baseline to 1 year was significantly smaller than 15% for sP-TM antigen and sP-TM activity in both EDTA and citrate (\( P < 0.001 \)), a result comparable to the interassay variation observed for control samples.

SP-TM activity and sP-TM antigen in normal human plasma

Blood samples from healthy blood donors, 50 men and 50 women, were obtained in EDTA-treated tubes and citrated tubes. The results are outlined as reference intervals (mean ± 2SD) in Table 1. Women tended to have lower plasma levels of sP-TM antigen (in agreement with earlier results [18]) and sP-TM activity than men.

To investigate the relationship between plasma sP-TM activity and sP-TM antigen measurements on baseline samples, the means from duplicate measurements on EDTA-treated and citrated plasma, as well as the means of all measurements on the same plasma samples, were fitted to the equation (sP-TM activity) = \( a + b \times (sP-TM\ \text{antigen}) \). The results are shown in Table 2 and the regression lines are shown in Fig. 2. For both men and women, there was a statistically significant correlation between sP-TM antigen and sP-TM activity (\( P < 0.001 \) for men and, \( P = 0.003 \) for women, both according to the mean from EDTA and citrated plasma). Interestingly, for this group of healthy blood donors, only 22% (men) and 16% (women) of the variation in sP-TM activity was explained by the variation in sP-TM antigen concentration. On average when sP-TM antigen increased 1 SEq mL\(^{-1}\) sP-TM activity was only increased by 0.34 SEq mL\(^{-1}\) (men) and 0.28 SEq mL\(^{-1}\) (women). We also fitted a new model comprising only females below the age of 60 years (we omitted data from five women aged > 60 years), and the new figures were almost identical to those in Table 2 (activity = 2.0 + 0.28 × antigen). We conclude that it is of almost no importance that we have included five women over the age of 60 years in our model. The group of women aged > 60 years are too few to be analyzed separately. In addition, we calculated the quotient (sP-TM activity)/

Table 1 Reference intervals (mean ± 2SD) for sP-TM antigen and sP-TM activity from duplicate measurements on plasma from two different blood sampling systems

<table>
<thead>
<tr>
<th></th>
<th>sP-TM antigen (SEq mL(^{-1}))</th>
<th>sP-TM activity (SEq mL(^{-1}))</th>
<th>sP-TM activity/ sP-TM antigen</th>
<th>sP-TM –antigen (SEq mL(^{-1}))</th>
<th>sP-TM activity/ sP-TM antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men</td>
<td>3.7–9.7</td>
<td>2.4–6.0</td>
<td>0.37–0.93</td>
<td>3.5–8.3</td>
<td>0.45–0.89</td>
</tr>
<tr>
<td>Women</td>
<td>3.1–9.1</td>
<td>2.2–5.4</td>
<td>0.32–0.96</td>
<td>3.2–7.2</td>
<td>0.38–0.98</td>
</tr>
</tbody>
</table>

1 ng Solulin = 1 SEq.

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For the men, median and ranges for this parameter were 0.65 (0.34–0.92) for EDTA and 0.67 (0.35–0.91) in citrate. For the women, the median and range were 0.64 (0.33–1.0) in EDTA and 0.68 (0.38–0.98) in citrated plasma.

Discussion

At higher than normal levels, circulating soluble TM fragments (measured as antigen levels, sP-TM antigen) have been considered an unspecific marker of endothelial cell injury. Earlier attempts to measure the functional activity of TM directly in plasma have failed [5], and a plausible explanation is that the total thrombin inhibitory capacity in normal plasma prevents the formation of the thrombin–TM complex and thereby protein C activation. Indirect measures of an anticoagulant cofactor activity associated with sP-TM antigen have been demonstrated in two studies. One clinical study demonstrated that the numbers of both serious hemorrhages and of total hemorrhages were significantly associated with increased levels of sP-TM antigen in plasma in patients on long-term anticoagulant therapy, and that the number of bleeding episodes increased exponentially through quartiles one to four of the TM distribution [19]. One small study demonstrated higher levels of sP-TM antigen in patients with acute bleeding duodenal ulcers compared with controls [20]. The hypothetical, and most obvious, explanation is that sP-TM antigen has cofactor activity in the thrombin-mediated activation of protein C. Higher-than-normal soluble TM activity will add to the anticoagulant milieu, which is not beneficial when combined with anticoagulant treatment. Another clinical study demonstrated a strong, graded inverse association between sP-TM antigen and coronary heart disease [21]. Again, this is consistent with sP-TM antigen having cofactor activity in the thrombin-mediated activation of protein C. Lower-than-normal activity of soluble TM fragments might conversely add to a procoagulant status that is superimposed on other risk factors in atherosclerosis. Finally, a recent prospective study failed to show any associations of age-adjusted sP-TM

**Table 2** Comparison of sP-TM activity and sP-TM antigen using the regression model (sP-TM activity) = a + b(sP-TM antigen)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>95% confidence interval</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>EDTA 2.8 1.7–3.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>citrate 1.6 0.50–2.6</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>mean 1.9 0.78–3.0</td>
<td>0.001</td>
</tr>
<tr>
<td>b</td>
<td>EDTA 0.21 0.050–0.37</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>citrate 0.39 0.22–0.57</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>mean 0.34 0.16–0.51</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>EDTA 2.4 1.5–3.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>citrate 1.8 0.69–2.9</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>mean 2.1 1.1–3.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>b</td>
<td>EDTA 0.22 0.070–0.38</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>citrate 0.32 0.11–0.53</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>mean 0.28 0.10–0.45</td>
<td>0.003</td>
</tr>
</tbody>
</table>

For males $R^2 = 11%$ (EDTA), 28% (citrate) and 22% (mean); for females $R^2 = 14%$ (EDTA), 15% (citrate) and 16% (mean).

(sP-TM antigen). For the men, median and ranges for this parameter were 0.65 (0.34–0.92) for EDTA and 0.67 (0.35–0.91) in citrate. For the women, the median and range were 0.64 (0.33–1.0) in EDTA and 0.68 (0.38–0.98) in citrated plasma.

![Fig. 2](image_url). The correlation between sP-TM antigen and sP-TM activity in citrated tubes (a), EDTA tubes (b) and the mean from both tubes (c) for men (●) and women (×). The parameters of the respective regression lines are outlined in Table 2.
antigen or the TM Ala455Val genotype with overall venous thrombosis or with any subtype of venous thrombosis [22]. In addition, it should be kept in mind that TM also has functions which are not mediated through its anticoagulant effect nor through the activation of the protein C pathway. It has been suggested that the lectin-like domain of TM interferes with polymorphonuclear leukocyte adhesion to endothelial cells by intracellular adhesion molecule-1-dependent and independent pathways. This could be another possible mechanism for the inverse association between soluble TM and cardiovascular disease [23,24].

To investigate the relationship between plasma sP-TM antigen and anticoagulant activity in healthy and diseased states in more detail, an assay is required to directly determine circulating TM cofactor activity in plasma. We have constructed such an assay aimed at measuring the thrombin cofactor activity of soluble TM fragments. Using this assay, we have demonstrated that these TM fragments maintain appreciable cofactor activity when released (degraded) from the endothelial cell surface into plasma. To ensure that this assay measures expected concentrations of TM in plasma and that the cofactor is not inhibited during the assay, the recovery of TM added to normal plasma was determined. The data demonstrate that the sP-TM activity assay reliably measures TM activity in plasma and that the experiment itself does not induce inhibition of TM. Furthermore, to test the specificity of the method, we pre-incubated the standard curve and three normal plasmas with an anti-TM antibody. The assay results were all comparable to the blank value. Finally, we have shown that healthy women have lower sP-TM activity than healthy men, according to their lower plasma sP-TM antigen levels.

We conclude that there is a statistically significant correlation between sTM antigen levels and sTM activity in healthy blood donors. Finally, and most interestingly, our investigation of healthy men and women demonstrates that for sP-TM antigen and sP-TM activity in plasma only 22% (men, mean from EDTA and citrate) and 16% (women, mean from EDTA and citrate) of the sP-TM activity variation is explained by the variation in sP-TM antigen levels. The most plausible explanation is that the variously sized TM fragments produced from the endothelial cell surface during the degradation process have activities ranging from full TM cofactor activity to zero. Some evidence for this hypothesis has been published [8]. The difference between females and males is hypothetically explained by sex-dependent degradation patterns. To demonstrate definitively the varying TM cofactor activity of the different TM fragments circulating in plasma in men and women, isolation, separation, and characterization of each fragment would be necessary.

In future investigations of the clinical importance of variations in circulating TM fragments as well as of TM mutations with impaired TM cofactor activity, an assay to measure sP-TM activity in plasma will be of the utmost importance. In addition, the assay will facilitate studies of sP-TM activity and its possible pathophysiological importance in various diseased states.

Acknowledgements

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