Measuring Tissue Factor (Factor III) Activity in Plasma

Chisako Fukuda,1 Kenji Iijima,2 and Katsumi Nakamura2

This is a method for measuring tissue factor (TF, Factor III, tissue thromboplastin) activity in plasma by using a chromogenic substrate. As pretreatment, the eguglobulin fraction of plasma was prepared by removing endogenous inhibitors and heated at 60 °C for 3 min to remove fibrinogen. This allowed us to measure the low TF activity in plasma that could not otherwise be measured. Neither phospholipids nor coagulation factors VII, IX, X, or Xa in the samples interfere. Within-run and day-to-day reproducibility were both good. The mean value obtained by this method for normal persons was 1.02 (SD 0.91) arbitrary units/L. A markedly high plasma TF activity of 20 arb. units/L or more was observed in patients with some types of disseminated intravascular coagulation.

Additional Keyphrases: coagulation · cancer · tissue thromboplastin · reference interval · disseminated intravascular coagulation

Tissue factor (TF), a lipoprotein, is present in brain, lung, and blood vessels and also in placental tissue and some tumor tissues (1). It consists of an apoprotein portion, containing the antigen common to various organs, and a phospholipid portion, which is required for expression of partial thromboplastin activity (2). After cell damage, the phospholipids of TF leak into the blood and form a complex with factor VII or VIIa in the presence of Ca2+. This directly activates factor X in the extrinsic pathway (3) and also factor IX (4, 5) in the intrinsic pathway (6).

TF is considered to be involved in causing disseminated intravascular coagulation (DIC) and to play an important role in abnormal coagulation and inflammatory reaction in patients with malignant tumors (7). In addition, its involvement also in various pathological conditions such as renal disease (8) and neurological disease (9) has been suggested.

TF has been measured in the urine (8), vascular endothelial cells (10), fibroblasts (11), monocytes (12), tumor cells (13), cerebrospinal fluid (9, 14), and amniotic fluid (15). Although the presence of TF as TF antigen in plasma has been confirmed—i.e., the apoprotein is present in the plasma of patients during surgery (16)—there are no reports on measuring TF activity in plasma. Currently available methods for measuring TF activity involve coagulation—Quick’s one-step method (17) and Nemerson’s two-step method (18)—and a method involving chromogenic synthetic substrates (14). In the present study, we measured the TF activity in plasma by using the chromogenic synthetic substrate S-2222.

Materials and Methods

Materials

Pooled normal plasma: Blood from 20 normal subjects was anticoagulated with citrate, final concentration 3.8 g/L.

Thromborel S (human placenta-derived TF, Behringwerke AG, Marburg, F.R.G.; lot no. 505433): the contents of one vial were dissolved in 4 mL of water before use.


Factor Xa (8.4 × 108 U/L; Daiichi Kagaku, Tokyo, Japan).

Prothrombin complex: PPSB (factor II, VII, IX, and X concentrates; Nippon Pharmaceutical Co., Tokyo, Japan).

Chromogenic synthetic substrate: S-2222 (Bz-Ile-Glu-Gly-Arg-pNA; KabVitrum AB, Stockholm, Sweden).

Subjects

Blood was sampled from 10 patients with an underlying disease such as malignant disease, infection, or blood disorder, who were diagnosed as having DIC by blood-coagulation tests and physical examination. The samples were anticoagulated with citrate and stored at −20 °C until assay. The number of samples from each patient differed according to circumstances. A set of 15 samples was analyzed concurrently.

Methods

Preparation of eguglobulin fraction: The eguglobulin fractions were prepared by titration of 10-fold-diluted (with distilled water) plasma with dilute (2.5 mL/L) acetic acid until an optimal pH was reached. The precipitated eguglobulins were removed by centrifugation, dissolved in barbital buffer (28 mmol/L, pH 7.35), and heated at 60 °C for 3 min to inactivate fibrinogen, then centrifuged at 1500 × g for 10 min. The supernatant fluid was used as the test sample.

Measurement of TF activity: Measurement of TF activity was based on the procedure of Hische et al. (14), who also used the chromogenic synthetic substrate S-2222.

After incubating 150 μL of the test sample with 300 μL of Tris buffer (200 mmol/L, pH 7.3) containing 15 mmol of CaCl2 per liter at 37 °C for 5 min, add 150 μL of the prothrombin complex diluted twofold with a 120 mmol/L solution of NaCl. After 10 min at 37 °C, take a 100-μL aliquot of the mixture and mix it with 600 μL of Tris buffer solution (700 mmol/L, pH 8.4) containing 2.5 mmol of EDTA per liter (37 °C) to terminate the reaction. Mix the reaction solution, now containing the newly formed factor Xa, with 100 μL of S-2222 and incubate the mixture at 37 °C for 3 min. Terminate this reaction by adding 300 μL of acetic acid diluted with an equal volume of water. Colorimetrically, at 405 nm, the p-nitroaniline released from the substrate.
Results

Analytical Variables

Proper incubation time: To determine this, we studied the changes with time of factor Xa formation in solutions containing TF diluted 50- and 200-fold and in 120 mmol/L NaCl solution. Up to 10 min, we observed no difference in formation of factor Xa among the three solutions. The reaction curve was linear, even for the higher-concentration TF solution. Thereafter, the slope of the curve was decreased in the case of the more highly concentrated solution of TF. Between 10 and 30 min, the absorbance increased slightly, even for the TF-free NaCl solution. We concluded that 10 min is the best incubation interval for measurement of factor Xa formation. Our results were similar to those described by Hische et al. (14).

Effects of pretreatment: The euglobulin fraction was prepared so as to remove inhibitors in plasma and heat-treated to inactivate fibrinogen, because these factors would interfere with the measurement. We determined the amounts of inhibitors and coagulation factors in the normal plasma samples before and after such treatment. Antithrombin III, \( \alpha_2 \)-plasmin inhibitor, \( \alpha_2 \)-macroglobulin, \( \alpha_1 \)-antitrypsin, fibrinogen, and factors II, V, and VIII—all of which were within the normal reference interval before the treatment—were decreased substantially or inactivated (Tables 1 and 2).

We compared the TF activity before and after the euglobulin fraction treatment, using six TF concentrations in plasma, obtained by mixing nine volumes of normal plasma with one volume of various dilutions of TF solution in 120 mmol/L NaCl. Before the treatment, the TF activity was decreased, probably owing to inhibitors, and it could not be measured in plasma containing TF diluted eight-fold or less. After the treatment, the low TF activity in the plasma containing TF diluted eight- to 32-fold could be determined, and the measured absorbance was related to this activity (Figure 1).

The absorbance of TF did not change after treatment at 60°C for 3 min, evidencing no decrease in the TF activity. After centrifugation, however, we saw a 25% decrease in its activity. Its mechanism remains to be clarified.

Effects of pH on preparation of the euglobulin fraction: The pH was varied from 4.6 to 7.2 during preparation of the euglobulin fraction. The TF activity measured in the euglobulin fractions obtained from normal plasma mixed with TF solution in a ratio of 9:1 showed no significant changes within this pH range (Figure 2).

Effects of storage of samples: Normal plasma mixed with TF (9:1 by vol) was divided into two aliquots. One was stored at 4°C, the other at -20°C. No significant changes were observed in the TF activity at either storage temperature until the fourth week.

Reproducibility: TF activity was measured in the normal plasma mixed with TF in a ratio of 9:1. Both within-run reproducibility and day-to-day reproducibility were good, the CVs being 2.5% \((n = 6)\) and 9.9% \((n = 12)\), respectively.

Effects of various substances in the measurement system: Thromborel S in the S-2222 reaction solution showed an absorbance of \(\leq 0.025\). The prothrombin complex showed a similar absorbance. Neither seemed to us to exceed the limits of error for the method.

TF activity was measured in the presence of phospholipid at various concentrations. Using phospholipid diluted two- and 50-fold with a 120 mmol/L solution of NaCl, we measured TF activities, using the same experimental procedures used to measure TF samples. Phospholipid exerted

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<th>Table 1. Changes in Concentrations of TF Inhibitors after Treatment of the Euglobulin Fraction</th>
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<td>Inhibitors</td>
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<td>Antithrombin III, %</td>
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<tr>
<td>( \alpha_2 )-Plasmin inhibitor, %</td>
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<td>( \alpha_2 )-Macroglobulin, g/L</td>
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<td>( \alpha_1 )-Antitrypsin, g/L</td>
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We measured the functional activity of antithrombin III and \( \alpha_2 \)-plasmin inhibitor by the chromogenic substrate method, the immunological activity of \( \alpha_2 \)-macroglobulin and \( \alpha_1 \)-antitrypsin by single radial immunodiffusion.

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<th>Table 2. Changes in Concentrations of Coagulation Factors after Heat Treatment</th>
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<td>Coagulation factors</td>
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<tr>
<td>Fibrinogen, g/L</td>
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<td>Prothrombin, %</td>
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<td>Factor V, %</td>
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<td>Factor VIII, %</td>
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We measured the functional activity of fibrinogen by the thrombin time method, the functional activity of prothrombin and factors V and VIII by the clotting method.

Fig. 1. Effects of euglobulin fraction preparation

Fig. 2. Effects of pH on TF activity in the euglobulin fraction preparation
negligible effects on the TF activities.

The $A_{405}$ for factor Xa at a final concentration of $1.26 \times 10^5$ units/L was 0.51 before the heat treatment, but 0.03 after the treatment in this measurement system, suggesting that, even when factor Xa is present in the sample, it is inactivated by heat treatment and so has no effect on S-2222.

We measured the TF activity in a 9:1 (by vol) mixture of a series of plasma samples deficient in factors VII, IX, or X and TF and compared it with the activity in normal plasma, to evaluate the effects of each of these coagulation factors on the measurement system. The $A_{405}$ was 0.700 for the normal plasma, 0.728 for the factor VII-deficient plasma, 0.617 for the factor IX-deficient plasma, and 0.731 for the factor X-deficient plasma. There was no significant difference between results for the normal plasma and any of the deficient plasmas. Evidently, factors VII, IX, or X in plasma samples do not affect the measurement system.

Normal values: The TF activity was measured in citrated plasma samples from 20 healthy subjects. We used the same arbitrary unit of TF activity as used by Hische et al. (14), 1 unit of TF being defined as the amount of TF that causes the generation of 1 unit of factor Xa per minute under standard conditions. One unit of factor Xa is defined as the amount of factor Xa catalyzing the cleavage of 1 mol of S-2222 per minute under standard conditions. The TF activity concentrations fell between 0 and 2.9 units/L, the mean being 1.02 (SD 0.91) units/L (Figure 3).

Plasma TF activity in DIC patients: TF activity was measured in 39 plasma samples from 10 DIC patients at the time of their first examination. The underlying disease was gastric cancer in two patients, primary liver cancer in two, metastatic liver cancer in two, pancreatic cancer in one, acute monocytic leukemia in one, pneumonia in one, and others in one. A TF activity of 20 units/L or more was observed in 19 samples from five patients (four with liver cancer and one with acute monocytic leukemia). A significant increase in the plasma TF activity was observed in five of the 10 patients (19 of 39 samples).

Discussion

The chromogenic synthetic substrate method is generally very sensitive and specific, and effects of inhibitors and fibrinogen in the sample often can be negated by high dilution of samples. There is no measurable TF activity in normal plasma; thus TF is considered to be released into the plasma after cell damage (19). Even when TF activity does appear in plasma, it is very low, and thus higher dilutions of plasma are not appropriate before measurement. Therefore, as pretreatment, we removed inhibitors from plasma by preparing the IgG globulin fraction and treating it with heat to avoid fibrin formation during measurement. Brain-derived TF is sensitive to heat, and a 30\% decrease in the activity after treatment at 50 °C for 2 h was reported (20). We heated TF derived from human placenta (rabbit-brain-derived TF for reference) at 60 °C for 3 min. No effects on TF activities of this heat treatment were observed, either in brain and placental preparations. After heating, TF was measured with the S-2222 solution to monitor the TF activity. Thus, after the above pretreatment the inhibitors antithrombin III and $\alpha_2$-plasmin inhibitor, and also $\alpha_2$-macroglobulin and fibrinogen were removed, and even TF activity so low as to cause no fibrin clot formation could be measured. In addition, assay of serial dilutions showed good linearity.

To evaluate the effects of other substances in the measurement system, we looked for TF activity in rabbit-brain-derived phospholipids, but found none. This supports the report that, when TF is separated into apoproteins and lipids, only the latter shows no TF activity (1).

Of the activators in plasma, factor Xa acted directly on synthetic substrate S-2222 and seemed to increase results in the measurement system. However, purified factor Xa added to the sample was completely inactivated after heating at 60 °C for 3 min. Therefore, even when factor Xa is present in the plasma sample, its effects on the measurement system may be removed by pretreatment.

Excessive amounts of factors VII, IX, and X were tested in the measurement system, and these factors in plasma samples do not appear to affect the measurement system, as was confirmed by the results of our evaluation in which we used plasma lacking each coagulation factor.

A significant increase in the plasma TF activity was observed in five of 10 patients (19 of 39 samples). These findings suggest that TF may be a trigger for DIC. In addition, TF can be a major index for evaluating the cause and kinetics of DIC along with changes in other coagulation and fibrinolysis factors associated with DIC.

References