Tissue Factor Pathway Inhibitor as a Universal Anticoagulant for Use in Clinical Laboratory Tests

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TSUJI, R., TATSUMI, N., HINO, M., NISHIOKA, T. and TAKUBO, T. Tissue Factor Pathway Inhibitor as a Universal Anticoagulant for Use in Clinical Laboratory Tests. Tohoku J. Exp. Med., 2001, 194 (3), 165-174 —— Tissue factor pathway inhibitor (TFPI) is a protease inhibitor of extrinsic coagulation. The present study investigates the possibility of utilizing TFPI as a universal anticoagulant in clinical laboratory tests. The optimal concentration of TFPI for use in clinical laboratory tests was found to be 1 μl TFPI/ml blood (100 mmol TFPI/ml blood); the subsequent analyses were conducted at this concentration. In hematological tests, complete blood cell count and differential white blood cell count were done with an automatic blood analyzer. The results except for platelet and white blood cell counts were similar for ethylenediaminetetraacetic acid (EDTA)-treated and TFPI-treated samples. The effects of TFPI on platelet count were more pronounced when blood samples were stored at 4°C than at room temperature. The effects of TFPI on cell morphology were evaluated by spreading blood samples into thin films and applying a Giemsa stain. The results showed that TFPI did not alter the morphology of blood cells. An automatic biochemical analyzer performed seventeen basic biochemical tests on serum samples and TFPI-treated plasma samples. The results of seventeen tests were comparable between TFPI-treated samples and EDTA-treated samples. The prothrombin time for TFPI-treated plasma samples was longer than that for citrated plasma samples. Nonetheless, in activated partial thromboplastin time tests, the addition of the reagent caused turbidity and partial coagulation, thus demonstrating that TFPI is not suitable for this assay. These findings suggest that although some tests cannot be performed with TFPI, this compound may be useful as a universal anticoagulant in the future. ——— tissue factor pathway inhibitor; anticoagulant; laboratory tests; ethylenediaminetetraacetic acid; whole blood

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Tissue factor pathway inhibitor (TFPI) is a 40 kDa glycoprotein of 276 amino acids with Kunitz-type domains and it can suppress the extrinsic pathway of tissue factor-induced coagulation (Lindahl et al. 1992; Sandset 1996; Mann et al. 1998). Its plasma concentration is normally at or below 100 ng/ml (Kokawa et al. 1995). Because this inhibitor has been found to attenuate thrombogenesis in rabbits with sepsis (Broze 1995; Park et al. 1997), research is being conducted to investigate its use in humans with sepsis. However, no effort has been made to utilize TFPI in clinical laboratory tests because most researchers focus on its pharmacological action and coagulation regulation function in vivo, but not on its use as an in vitro test reagent.

Various attempts are being made to improve the speed, economics, and quality of clinical laboratory tests (Guder et al. 1996; Tatsumi et al. 1999) through the use of a comprehensive automatic analysis system. Automation should make it possible to perform different tests simultaneously with small amounts of blood, eliminating the need to collect large quantities of blood in several test tubes to carry out different tests. Thus, much volume of blood is required to patients to receive laboratory tests, such as 2 ml ethylenediaminetetraacetic acid (EDTA) blood for hematology tests and 10 ml or more blood for chemistry tests. Nevertheless, large amounts of blood are being disposed of after each automated laboratory test.

If an appropriate anticoagulant is developed, hematological and chemical tests can be performed at the same time, thus reducing the amount of blood collected from patients. Consequently, there have been studies on several universal anticoagulants, including fractionated heparins, in recent years (Tamada et al. 1998; Kawamoto et al. 2000; Kinoshita et al. 2000; Kumura et al. 2000a, b, c; Narita et al. 2000). The present study investigates the use of TFPI as a universal anticoagulant in clinical laboratory tests.

**MATERIALS AND METHODS**

**Blood samples**

After obtaining informed consent from healthy volunteers, venous blood samples were collected. For comparison, EDTA-treated samples were used in hematological tests, serum samples were used in biochemical tests, and citrated blood samples were used in coagulation tests. Blood samples were either collected in plastic syringes and transferred to plastic test tubes containing TFPI or collected in commercially available vacuum blood collection tubes containing TFPI (Terumo Corp., Tokyo).

**Tissue factor pathway inhibitor stock solution**

To make the TFPI stock solution, 199.3 mmol recombinant TFPI (rTFPI; Chemo-Sciro-Therapeutic Research Institute, Kumamoto) was dissolved in 20 mmol/liter of citrate buffer and 0.10 mmol/liter of NaCl (pH 7.4). The stock solution was dissolved with whole blood to make an anticoagulated blood with a definite concentration of TFPI. The TFPI reagent was divided into small containers and stored at −20°C, to be further divided into small test tubes before use.

**Anticoagulation experiment**

From the stock solution, three concentrations of TFPI (1, 5, or 10 μl TFPI/ml blood) were placed in plastic test tubes and fresh venous blood was added to each tube with a sterile plastic syringe. The contents were mixed by gently inverting the tubes, then the tubes were placed in constant-temperature baths (4°C, 25°C, or 37°C). At predetermined times, the test tubes were tilted to macroscopically assess coagulation.

**Hematological tests**

Complete blood counts and differential white blood cell counts were performed on TFPI-treated and EDTA-treated samples by an
automatic blood analyzer (NE-8000; Sysmex Corp, Kobe). Blood samples containing 1, 5, or 10 µl/ml of TFPI stored at 4°C or 25°C were analyzed at 0, 6, and 24 hours after blood collection (n = 7).

**Blood morphology tests**

The TFPI-treated blood was spread into a thin film over a glass microscope slide and stained with May-Grünwald-Giemsa stain. Then the morphology of the stained cells was examined under a light microscope at magnifications of ×100, ×400, and ×1000 (oil immersion).

**Biochemical tests**

The TFPI-treated blood was centrifuged at 3000 rpm (2500 × g) for 15 minutes and the resulting plasma was transferred to an automatic biochemical analyzer (Model 7170; Hitachi, Tokyo) which then performed 17 basic biochemical tests. Serum samples that were collected and prepared at the same time as the TFPI-treated blood were analyzed for comparison.

**Coagulation tests**

Simplastin Excel® reagent (Organon Teknika Corp., Durham, NC, USA) was used in the prothrombin time tests and Automated APTT (Organon Teknika) was used in the activated partial thromboplastin time tests. These tests were performed by conventional methods.

**Statistical analyses**

Paired t-tests were performed with Microsoft® Excel 98 (Microsoft Japan, Tokyo).

**RESULTS**

**Anticoagulation experiment**

The TFPI at all three concentrations (1, 5, and 10 µl/ml blood) maintained the fluidity of blood immediately after collection. The samples were then stored at 4°C, 25°C, or 37°C and coagulation was assessed macroscopically at predetermined times. At each concentration and temperature, coagulation was not observed for the first 6 hours. At 37°C, hemolysis occurred in the plasma portion of each sample after 6 hours and coagulation was confirmed in every sample after 24 hours. At 25°C, partial coagulation was confirmed in 4 of 7 samples containing 1 µl TFPI/ml blood after 24 hours, however, no coagulation was seen at this temperature in samples with the higher concentrations of TFPI. One µl of TFPI/ml blood could be judged to promise steady and stable hematological testing within 6 hours after blood collection as an anticoagulant. On the basis of these findings, a mixing ratio of 1 µl TFPI: 1 ml blood was chosen as its practical concentration in laboratory use, and the subsequent tests were performed at this concentration.

**Hematological tests**

The analyzer freely suctioned TFPI-treated blood samples except for the previously reported cases of coagulation. Complete blood counts were performed on TFPI-treated and EDTA-treated samples (Table 1). Regardless of storage temperature, the levels of hemoglobin, red blood cells, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration remained fairly constant for the first 6 hours (Fig. 1). However, in those samples stored at 25°C for 24 hours, the levels of mean corpuscular volume and hematocrit increased; no change was seen in the samples stored at 4°C for 24 hours. The white blood cell count for TFPI-treated samples was higher than that for EDTA-treated samples immediately after blood collection and comparable after 6 hours at 25°C (Fig. 2). At 4°C, the white blood cell count for TFPI-treated samples was lower than that for EDTA-treated samples. The platelet count for TFPI-treated samples was less than half of that for EDTA-treated samples immediately after blood collection (Fig. 2). The platelet count
Table 1. Complete blood count immediately after blood collection

<table>
<thead>
<tr>
<th>Item</th>
<th>EDTA-2K</th>
<th>TFPI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 μl</td>
<td>5 μl</td>
</tr>
<tr>
<td>WBC (10^9/μl)</td>
<td>44.0 ± 9.8</td>
<td>55.9 ± 11.0</td>
</tr>
<tr>
<td>RBC (10^6/μl)</td>
<td>461 ± 44</td>
<td>463 ± 43</td>
</tr>
<tr>
<td>Hgb (g/100 ml)</td>
<td>14.8 ± 1.5</td>
<td>14.8 ± 1.5</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>42.5 ± 4.1</td>
<td>42.3 ± 4.2</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>92.1 ± 1.7</td>
<td>91.3 ± 1.8</td>
</tr>
<tr>
<td>Plt (10^3/μl)</td>
<td>22.2 ± 4.8</td>
<td>8.1 ± 2.9</td>
</tr>
</tbody>
</table>

Each value shows mean ± s.d. (n = 6)
EDTA, ethylenediaminetetraacetic acid; TFPI, tissue factor pathway inhibitor; WBC, white blood cell count; RBC, red blood cell count; Hgb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; Plt, platelet count.

![Graphs showing changes over time](image)

Fig. 1. Influences of TFPI on determination for red cell parameters.
- - - , ethylenediaminetetraacetic acid; △ - △, tissue factor pathway inhibitor (TFPI) 1 μl;
- - - , TFPI 5 μl; ○ - ○, TFPI 10 μl. A, 25°C; B, 4°C.

For TFPI-treated samples stored at 25°C was about 75% of that for EDTA-treated samples stored at 25°C, but at 4°C, the decrease in the platelet count was even more pronounced. Because the automatic analyzer counts platelet aggregates as white blood cells (Rowan 1991), decreases in the platelet count showed up as increases in the white blood cell count at 25°C and decreases in both platelet and white blood cell counts over time at 4°C.

In the differential white blood cell count, the levels of lymphocytes and eosinophils increased and the levels of neutrophils and monocytes decreased in both TFPI-treated and EDTA-treated samples stored at 4°C relative to their levels immediately after blood collection.
Fig. 2. Influences of TFPI on enumeration of white cells and platelets.

- , ethylenediaminetetraacetic acid; △ — △, tissue factor pathway inhibitor (TFPI) 1 μl; ○ — ○, TFPI 5 μl; ◇ — ◇, TFPI 10 μl. A, 25°C; B, 4°C.

(Fig. 3). On the scattergram, granulocyte fraction border was dislocated from the normal gating area and the analyzer displayed a “platelet aggregation” message. Automatic differentiation is heavily dependent on the computer program of the analyzers (Pierre 1991; Groner and Simson 1995). Chronological changes in platelet aggregation may affect automatic fraction ratios and thus skew results.

To investigate the effects of TFPI on blood cell morphology, blood samples were stored at 4°C or 25°C for 24 hours, then spread into thin films on glass microscope slides and stained with a Giemsa stain. The cellular structure of the stained cells was then ascertained under a light microscope. The results showed that TFPI had no notable effects on the morphologies of the red and white blood cells at both temperatures. However, many large and small aggregates of platelets were observed, but not of white cells and red cells. There was no white blood cell aggregates.

Biochemical tests

The automatic analyzer performed 18 basic clinical laboratory tests on serum samples and TFPI-treated plasma samples. The results were comparable for 18 tests, but there were significant differences in the levels of some items between the two groups although most of mean values were close (Table 2).

Coagulation tests

Citrated plasma samples and TFPI-treated plasma samples were analyzed. The prothrombin times for TFPI-treated plasma samples were noticeably longer than those for citrated plasma samples in a concentration-dependent manner (Table 3). In the activated partial thromboplastin time tests, as soon as the appropriate reagent was added, fibrin precipitated in the TFPI-treated plasma samples, creating turbid-
Fig. 3. Percent changes in percent neutrophils (Neu%) and percent lymphocytes (Lym%).
- - •, ethylenediaminetetraacetic acid; △ — △, tissue factor pathway inhibitor (TFPI) 1 μl;
□ — □, TFPI 5 μl; ○ — ○, TFPI 10 μl. A, 25°C; B, 4°C.

DiscusSion

Tissue factor pathway inhibitor exists in vivo in vascular endothelial cells, and when these cells are damaged, the syntheses of TFPI and tissue factor increase. These compounds are then released from the cells to regulate the entire coagulation reaction on the cell membrane by balancing TFPI-induced anticoagulation and tissue factor-induced coagulation (Lindahl et al. 1992; Kokawa et al. 1995; Sandset 1996; Mann et al. 1998). Tissue factor pathway inhibitor has three Kunitz-type domains: the first domain binds with the tissue factor-coagulation factor VII complex; the second domain binds with coagulation factor Xa (Lindahl et al. 1992; Kokawa et al. 1995; Sandset 1996; Kazama 1997; Mann et al. 1998) to express its inhibitory activity, but the function of the third domain has not been yet clarified. In blood, it is quickly degraded and inactivated. Recent advances in genetic recombination allowed us to use recombinant TFPI (Kazama 1997) with anticoagulation activity in the present study.

A desired condition for a universal anticoagulant is to get steady unclotability until all of laboratory tests are completed. Usually laboratory specimens are stored at or below room temperature. Twenty-four hours after blood collection, some of specimens containing 1 μl TFPI/ml blood showed partial coagulation, most likely because the TFPI would be degraded in whole blood, thus reducing its anticoagulation activity.

The suitable concentration of TFPI was judged to be 1-10 μl/ml blood, which is lesser concentration than that of heparin (1.0-1.5 mg/ml blood) or EDTA (1.5-2.2 mg/ml blood). To reduce cell damage, the mixing ratio of 1 μl
TABLE 2. Data comparison on chemistry tests

<table>
<thead>
<tr>
<th>Item</th>
<th>Serum value</th>
<th>Value of TFPI plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein (g/100 ml)</td>
<td>7.5±0.3</td>
<td>7.6±0.3**</td>
</tr>
<tr>
<td>Albumin (g/100 ml)</td>
<td>4.8±0.1</td>
<td>4.7±0.2**</td>
</tr>
<tr>
<td>Total bilirubin (mg/100 ml)</td>
<td>0.5±0.2</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>Triglyceride (mg/100 ml)</td>
<td>69±27</td>
<td>65±23</td>
</tr>
<tr>
<td>Total cholesterol (mg/100 ml)</td>
<td>186±58</td>
<td>181±57*</td>
</tr>
<tr>
<td>Urea nitrogen (mg/100 ml)</td>
<td>12.1±1.9</td>
<td>12.1±1.7</td>
</tr>
<tr>
<td>Creatinine (mg/100 ml)</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>Uric acid (mg/100 ml)</td>
<td>3.9±0.8</td>
<td>3.9±0.9</td>
</tr>
<tr>
<td>Sodium (mEq/liter)</td>
<td>144±2</td>
<td>143±1</td>
</tr>
<tr>
<td>Chlorine (mEq/liter)</td>
<td>107±2</td>
<td>108±2</td>
</tr>
<tr>
<td>Potassium (mEq/liter)</td>
<td>4.2±0.4</td>
<td>3.9±0.4***</td>
</tr>
<tr>
<td>AST (IU/liter·37°C⁻¹)</td>
<td>15±4</td>
<td>16±4</td>
</tr>
<tr>
<td>ALT (IU/liter·37°C⁻¹)</td>
<td>10±3</td>
<td>10±3</td>
</tr>
<tr>
<td>Lactate dehydrogenase (IU/liter·37°C⁻¹)</td>
<td>312±52</td>
<td>380±55***</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU/liter·37°C⁻¹)</td>
<td>116±24</td>
<td>113±24***</td>
</tr>
<tr>
<td>Leucine aminopeptidase (IU/liter·37°C⁻¹)</td>
<td>100±11</td>
<td>99±10</td>
</tr>
<tr>
<td>γ-glutamyl transpeptidase (IU/liter·37°C⁻¹)</td>
<td>12±2</td>
<td>11±2***</td>
</tr>
<tr>
<td>Cholinesterase (IU/liter·37°C⁻¹)</td>
<td>274±47</td>
<td>268±46***</td>
</tr>
</tbody>
</table>

Each value shows mean±s.d. (n=8)
TFPI, tissue factor pathway inhibitor; AST, α-Aspartate: 2-oxoglutarate aminotransferase; ALT, α-Alanine: 2-oxoglutarate aminotransferase.
*p<0.05. **p<0.01. ***p<0.001.

TABLE 3. Coagulation test results

<table>
<thead>
<tr>
<th>Item</th>
<th>Citrated plasma (second)</th>
<th>TFPI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 μl plasma (second)</td>
<td>5 μl plasma (second)</td>
</tr>
<tr>
<td>PT</td>
<td>12.9±0.6</td>
<td>83.5±7.7</td>
</tr>
<tr>
<td>APTT</td>
<td>42±6</td>
<td>Coagulated</td>
</tr>
</tbody>
</table>

Each value shows mean±s.d. (n=6)
TFPI, tissue factor pathway inhibitor; PT, prothrombin time; APTT, activated partial thromboplastin time.

TFPI/ml blood was chosen for the emergency laboratory use. In the present study, a solution of TFPI was mixed with blood samples, but the results were the same when desiccated TFPI was mixed with blood samples. The amount of TFPI solution was so small compared to blood volume to be mixed that the low concentration contributes to reduce reagent cost in the blood container.

Advantageous point of whole blood testing is that both hematology tests and chemistry test can be conducted with the same specimens. Modern fully-automated hematology analyzers implement complete blood counting and automated white cell differential analysis in a short minutes with less than 100 μl of whole blood anti-coagulated with EDTA. Whole blood with TFPI behaved in the similar manner of EDTA-treated blood for complete blood count and automated white cell differential, although the differential is a matter of some subtlety. However, the automatic blood anal-
yzer displayed a decrease in platelet count with a message indicating platelet aggregation, which would affect the differential white blood cell count. This decrease in platelet count was more pronounced in samples stored at 4°C than in samples stored at room temperature. A similar decrease in platelet count has been seen in blood treated with heparin (van Assendelft and Simmons 1995), where it is generally thought that heparin induces aggregation by the potent negative charge of its molecular structure. Because TFPI also has a strong negative charge on its C-terminus (Wesselschmidt et al. 1992), this charge could be closely involved in aggregation. Nonetheless, heparin-induced aggregation in vitro may not necessarily be comparable to TFPI-induced aggregation in vitro.

In recent years, the morphology of blood cells has been examined by preparing thin film specimens of EDTA-treated blood and staining them. In this study, TFPI was used like EDTA to prepare specimens and the morphology of the resulting cells was analyzed under a microscope. TFPI treatment fortunately showed no effects on morphology of red cells and white cells on Giemsa stained-films except for platelet aggregate formation at high TFPI concentrations. However, no reports have indicated that TFPI inhibits thrombogenesis and enhances platelet activation. Therefore, aggregation could be induced when the negative charge on the C-terminus of TFPI alters the glycoprotein structure of the platelet membrane (Kamikubo et al. 1997). Because platelet count decreases with time, it is necessary to complete all tests possibly soon after blood collection or to add an anti-platelet agent, anti-gpIIb/IIIa antibody (Reinhart et al. 1990) or kanamycin (Sakurai et al. 1997).

Increase in white cell count was induced by TFPI because of difficulty in separation between white cell and platelet areas because of dislocation of the white cell cluster close to the platelet area on the scattergram, but the increase in white cell count could be corrected by changing the gating and counting program in the hematology analyzer.

When biochemical tests are performed on TFPI plasma samples, all of items tested showed close values except the level of lactate dehydrogenase, although some of items showed significant but small differences. The high elevation of lactate dehydrogenase would caused by strong platelet aggregation.

All of anticoagulants except sodium citrate cannot be used in coagulation tests. As we expected, the prothrombin time for TFPI-treated samples was slightly greater than that for citrated samples. Probably some of constituents in the prothrombin time reagent could help to interfere the inhibitory action of TFPI to the extrinsic coagulation pathway. In activated partial thromboplastin time tests, as soon as the reagent was added, turbidity and coagulation were confirmed, demonstrating that TFPI cannot be used to investigate intrinsic coagulation abnormalities. Nonetheless, prothrombin time is the most frequently measured parameter in coagulation tests, and if the reference level can be changed to fit our TFPI treated plasma, prothrombin time can be measured by treated TFPI, thus expanding the range of TFPI applications.

It is a current problem for the medical laboratory to reduce blood requirement for patients who are receiving laboratory tests, because various types of blood, serum, plasma and whole blood, are generally required for laboratory tests to meet each types of laboratory instruments. Another problem is that currently-used anticoagulants such as EDTA, heparin, sodium fluoride and sodium citrate, have their own difficulties on laboratory testing (van Assendelft and Simmons 1995). This is the reason that an ideal universal anticoagulant is required. Universal anticoagulants must satisfy the following conditions: potent and consistent anticoagulation activity at a low concentration for a long period of time; readily dissolved in blood; minimal effects on the
morphology of blood cells; minimal effects on blood pH; active in blood collection tubes; useful in both hematological and chemical tests with reliable results; non-toxic to the human body; easy to use; stable in long-term storage; environmentally safe; and inexpensive.

So far, the following compounds have been investigated as possible universal anticoagulants: ethyleneglycoltetraacetic acid (Narita et al. 2000), sodium fluoride (Narita et al. 2000) and citric acid-theophylline-adenosine-dipyrimidamole (Reinhart et al. 1990) as chelating agents, fractionated heparins with anti-Xa and anti-thrombin activities (Kawamoto et al. 2000), hirudin with potent anti-Xa activity (Kumura et al. 2000a), argatroban (Kumura et al. 2000b), DX-9065a (Kumura et al. 2000c), and isoprene sulfate, which is a synthetic polymer that acts as a fibrinogen conversion inhibitor (Tamada et al. 1998; Kinoshita et al. 2000). Of these, DX-9065a appears to be the most promising universal anticoagulant. TPFI has several advantageous points as a universal anticoagulant; it is a non-toxic physiologic substance, and the concentration to be used as a anticoagulant is lower than those of EDTA and heparin.

In conclusion, TFPI would be a suitable clinical laboratory test reagent: 1) its potent anticoagulation activity is maintained for more than 6 hours at a low concentration; 2) like heparin, this peptide possesses physiological activity; 3) it is readily soluble in water; 4) it is not toxic to humans; 5) it does not pollute the environment; 6) it can be produced industrially in large quantities at low cost if it becomes to be used widely; and 7) a smaller, shorter and more consolidated laboratory system than the current one can be constructed by the use of TFPI. Because TFPI as a clinical laboratory test material has diverse characteristics, further research must be conducted on pathological samples. The results of the present study nevertheless show a high likelihood of successfully utilizing TFPI as a clinical labora-

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References


