Delayed Treatment with Recombinant Human Tissue Factor Pathway Inhibitor Improves Survival in Rabbits with Gram-Negative Peritonitis

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To determine whether treatment with recombinant human tissue factor pathway inhibitor (TFPI), an inhibitor of the extrinsic coagulation pathway, can improve survival in a clinically relevant model of gram-negative sepsis, rabbits were given an intraperitoneal inoculation of a suspension containing hemoglobin (40 μg/mL), porcine mucin (150 μg/mL), and viable Escherichia coli O18:K1 (1.0 ± 0.5 × 10^8 cfu/kg). Treatment with gentamicin (5 mg/kg every 12 h for five doses) was instituted 4 h after induction of peritonitis. At the same time point, rabbits were randomized to receive a 24-h infusion of vehicle or one of three different doses of TFPI. Treatment groups, 7-day survival rates, and significance versus control were as follows: control, 1 of 20; TFPILOW DOSE (0.1 mg/kg, then 1 μg/kg/min), 3 of 12 (P = .14); TFPI MIDE OSE (0.5 mg/kg, then 5 μg/kg/min), 7 of 12 (P = .002); TFPI HIGH DOSE (10 mg/kg, then 10 μg/kg/min), 4 of 13 (P = .04). Thus, delayed treatment with TFPI improves survival in septic rabbits.

Derangements in clotting and coagulation are common complications of gram-negative sepsis [1]. Thrombocytopenia occurs in 18%–74% of patients with sepsis [2–5]. Frank disseminated intravascular coagulation (DIC), while less common than simple thrombocytopenia, is nevertheless a very serious and fairly frequent occurrence in patients with overwhelming gram-negative infections [6–10]. Severe abnormalities in coagulation have been implicated as prognostic variables predicting a poor outcome in patients with sepsis [5, 8, 11]. Although the mechanisms responsible for thrombocytopenia and/or DIC in sepsis remain to be elucidated completely, currently available data suggest that events initiated by tissue factor are particularly important [12].

Tissue factor is a 263-aa transmembrane glycoprotein [13]. The expression of tissue factor on vascular endothelial cells and cells of the monocyte/macrophage lineage is increased following exposure to lipopolysaccharide, a component of the outer cell wall of gram-negative bacteria, and/or certain proinflammatory cytokines, notably tumor necrosis factor-α and interleukin-1, which have been implicated in the pathogenesis of sepsis [14–18]. Tissue factor binds and activates factor VIIa, leading to the formation of a tissue factor–factor VIIa complex that is capable of activating both factors X and IX [13].

Tissue factor pathway inhibitor (TFPI) is a naturally occurring inhibitor of tissue factor–initiated coagulation [19]. TFPI, also known as extrinsic pathway inhibitor or lipoprotein-associated coagulation inhibitor, is a member of a large family of serine protease inhibitors (Kunitz-type protease inhibitors). TFPI inhibits the tissue factor–factor VIIa complex via an ordered sequence of reactions in which the active site of factor Xa binds to TFPI and then the factor Xa–TFPI complex, in the presence of ionized calcium and factor Xa, binds the tissue factor–factor VIIa complex [19]. In humans and other species, the normal circulating concentration of TFPI is ~100 ng/mL [19]. Circulating TFPI levels are reportedly slightly elevated in patients with sepsis [20, 21] and the acute respiratory distress syndrome [22], although low plasma TFPI levels have been detected in some patients with gram-negative bacteremia and DIC [20].

TFPI has been sequenced and cloned [23]. In a primate model of lethal gram-negative bacteremia, treatment with recombinant TFPI has been shown to significantly improve survival, even when administration of the protein is delayed for several hours after the induction of sepsis [24, 25]. In the animal model used for these studies, septic shock was induced by intravenously infusing an enormous number (>10^10 viable cfu/kg) of Escherichia coli over a 2-h period. In the clinical setting, however, sepsis typically evolves more gradually as a result of the proliferation of microorganisms in vivo at a nidus of infection [26]. The present study, therefore, evaluated the therapeutic potential of recombinant TFPI as an adjuvant to conventional antimicrobial chemotherapy in an animal model of lethal gram-negative sepsis characterized by the presence of a focus of infection initiated by a relatively small inoculum of viable organisms.
Materials and Methods

Animals. New Zealand White rabbits (11–14 weeks old; 2.8–3.1 kg; Millbrook Associates, Amherst, MA) were maintained in individual wire cages in a temperature-controlled room with a 12-h light-dark cycle and permitted free access to water and a standard chow.

Bacteria. For these studies, we used a species and strain of bacteria, E. coli O18:K1, that is frequently isolated from patients with gram-negative bacteremia [26]. The initial inoculum was a gift from H. Shaw Warren (Massachusetts General Hospital, Boston). This isolate is susceptible to gentamicin in vitro. For each experiment, a frozen aliquot of the organisms was thawed and used to inoculate 125 mL of medium (Bacto Nutrient Broth; Difco, Detroit). After being incubated with shaking at 37°C for 15 h, the broth was centrifuged (2500 g at 4°C for 10 min). The pellet was resuspended in 50 mL of normal saline. After two more cycles of centrifugation and resuspension, the final concentration of bacteria was estimated nephelometrically by measuring optical density at 620 nm and comparing the result with a previously generated standard curve plotting optical density against viable colony-forming units.

Recombinant human TFPI. Nonglycosylated TFPI containing an additional alanine residue at the amino terminus of the wild type molecule was obtained from Chiron/Searle (Emeryville, CA). The protein was expressed in E. coli as previously described [27] and was formulated in a buffer consisting of 20 mM citrate, 200 mM arginine, 150 mM NaCl, and 0.01% Tween 80 (pH 6.0). The same buffer, but without TFPI, was used throughout the experiments as the control solution. Endotoxin levels in the TFPI preparations were <0.8 EU/mL.

Surgical preparation. Rabbits were anesthetized with intramuscular ketamine hydrochloride (35 mg/kg) and xylazine (5 mg/kg). Via a cervical cutdown, polyethylene catheters were positioned in a carotid artery and jugular vein. The catheters were exteriorized on the dorsal surface of the neck and secured in place with 3-0 suture. The catheters were flushed with 2 mL of heparinized saline (0.5 U/mL) and capped. Via a 1-cm midline laparotomy incision, the peritoneal cavity was inoculated with 10 mL of a treated and control groups. All other results were analyzed by one-tailed Student’s t-test. Survival was compared with the Breslow test. Fisher’s exact test was used to determine the significance of differences in survival at 7 days between groups. Statistical analyses.

Experimental design. We typically conducted one experiment per week. For each experiment, 6 rabbits, carefully matched for age and weight, were prepared and studied as a group. In each experiment, 2 of the 6 animals always received the buffer formulation without TFPI and served as concurrent controls. Overall, we conducted three series of experiments.

In the first series, rabbits were randomly allocated to either TFPI-treated or control groups. The treated rabbits (n = 11) received an initial bolus of TFPI (1.0 mg/kg) over 15 min at 6 h after the induction of sepsis. The bolus of TFPI was followed by a continuous infusion of the agent (10 μg/kg/min) for 12 h. Controls (n = 10) received similar volumes (bolus plus infusion) of the vehicle for TFPI.

In the second series of experiments, the treatment group (n = 8) was given a bolus of TFPI (1 mg/kg) over 15 min at 4 h after induction of sepsis, followed by a continuous infusion of 10 μg/kg/min for 24 h. The control rabbits (n = 8) received a similar amount of the vehicle.

In the third series of experiments, we studied 4 groups of animals: control (n = 20), TFPILOW DOSE (n = 12), TFPI MIDDOS E (n = 12), and TFPIHIGH DOSE (n = 13). All groups received a bolus of either buffer (control group) or TFPI at 4 h after induction of sepsis, followed by a continuous infusion of the vehicle or TFPI for 24 h. The TFPILOW DOSE group received a 0.1-mg/kg bolus of TFPI followed by an infusion of 1 μg/kg/min. The TFPI MIDDOS E group received a 0.5-mg/kg bolus of TFPI followed by an infusion at 5 μg/kg/min. The TFPIHIGH DOSE group received a 10-mg/kg bolus of TFPI followed by 10 μg/kg/min.

In all three series of experiments, both control and TFPI-treated rabbits received intravenous gentamicin (5 mg/kg/dose) every 12 h for five doses or until death. In the first series, treatment with gentamicin was initiated at 6 h after induction of sepsis, whereas in the second and third series of experiments, treatment with the antibiotic was started at 4 h.

Hematologic and biochemical assays. Blood lactate concentrations were determined by use of a commercial analyzer (Yellow Spring Instruments, Yellow Springs, OH). Circulating white blood cell (WBC) and platelet counts were determined by use of a particle counter (Coulter Electronics, Miami). Arterial blood gases were measured with an analyzer from Instrumentation Laboratories (Lexington, MA). Assays for various parameters of coagulation and plasma protein concentrations were determined by use of a device from Instrumentation Laboratories (model ACL 3000+).

Statistical analyses. Except for survival data, results are presented as means ± SEs. Kaplan–Meier survival curves were analyzed with the Breslow test. Fisher’s exact test was used to determine the significance of differences in survival at 7 days between treated and control groups. All other results were analyzed by one-way or two-way (group and time) repeated-measures analysis of variance, with post hoc analyses being done with the Student-Newman-Keuls test. Results were considered significant for P < .05.

Results

Experiment 1. In this experiment, peritonitis was induced at 0 h, and treatment with gentamicin plus either vehicle or TFPI (1 mg/kg bolus plus 10 μg/kg/min for 12 h) was started at 6 h. As evident from the pooled data for all animals (i.e., both vehicle- and TFPI-treated) shown in table 1, the rabbits manifested obvious signs of sepsis, such as leukopenia, hyperlactatemia, and metabolic acidosis, by the time treatment was initiated. Nevertheless, as shown in figure 1, treatment with TFPI significantly prolonged mean survival time from 38 h in controls to 81 h in drug-treated rabbits (P = .05). At 7 days, survival was 1 of 10 in vehicle-treated rabbits and 4 of 11 in TFPI-treated rabbits.
Table 1. Laboratory values in septic rabbits at 0, 3, and 6 h after induction of sepsis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time (h)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>PaO2 (mm Hg)</td>
<td>72 ± 15</td>
</tr>
<tr>
<td>PacO2 (mm Hg)</td>
<td>43 ± 4</td>
</tr>
<tr>
<td>pHa</td>
<td>7.40 ± 0.01</td>
</tr>
<tr>
<td>HCO₃ (mEq)</td>
<td>26.7 ± 1.8</td>
</tr>
<tr>
<td>BE (mEq)</td>
<td>2.2 ± 1.2</td>
</tr>
<tr>
<td>Lactate (mM)</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>WBC count (cells/μL)</td>
<td>4400 ± 390</td>
</tr>
</tbody>
</table>

NOTE: All values were obtained before initiating treatment with vehicle or TFPI. PaO2, PacO2, partial arterial pressure of O2 and CO2, respectively; WBC, white blood cells; pHa, arterial pH; BE, base excess.

* *P < .05 vs. baseline value (analysis of variance and Student-Newman-Keuls test).

Figure 1. Effect of treatment with TFPI (solid line) or vehicle (broken line) on survival in rabbits with gram-negative peritonitis. Sepsis was induced at 0 h and treatment was initiated at 6 h. * *P < .05 vs. controls (Breslow test).

Figure 2. Effect of treatment with TFPI (solid line) or vehicle (broken line) on survival in rabbits with gram-negative peritonitis. Sepsis was induced at 0 h and treatment was initiated at 4 h. * *P < .05 vs. controls (Breslow test).

Figure 3. Effect of treatment with TFPI (3 different dosing regimens) or vehicle on survival in rabbits with gram-negative peritonitis. Sepsis was induced at 0 h and treatment was initiated at 4 h. Groups are as follows: short dashed rule, TFPILOW DOSE; long dashed rule, TFPIHIGH DOSE; solid rule, TFPIHIGH DOSE; alternating short and long dashes, control. * Significant (P < .05) difference in mean survival time between indicated group and controls (Breslow test).

Experiment 2. The goal of the second experiment was to determine if earlier and more prolonged treatment with TFPI (i.e., 1 mg/kg bolus plus 10 μg/kg/min for 24 h started at 4 h) would afford a greater improvement in survival in rabbits with gram-negative peritonitis than was observed in experiment 1. As in the previous experiment, rabbits manifested signs of sepsis (leukopenia, respiratory alkalosis) at the time treatment with antibiotics plus vehicle or TFPI was initiated. Treatment with TFPI dramatically improved 7-day survival from 0 of 8 in controls to 7 (87.5%) of 8 in animals treated with the recombinant protein (P = .006; figure 2). Mean survival time was significantly increased from 48 h in controls to 149 h in TFPI-treated rabbits (P = .001).

Experiment 3. There were two goals of the third experiment. First, we sought to determine if doses of TFPI lower than 1 mg/kg bolus plus 10 μg/kg/min would be sufficient to improve survival in rabbits with gram-negative sepsis. Second, we sought to obtain more detailed information regarding the effects of treatment with TFPI in sepsis by measuring several physiologic parameters over time in animals treated with either vehicle or the experimental agent. Accordingly, rabbits were treated beginning at 4 h with gentamicin plus either vehicle or one of three different doses of TFPI. As shown in figure 3, all three doses of TFPI significantly improved survival compared with that in concurrent vehicle-treated controls. Seven-day survival rates for the 4 study groups were as follows: vehicle controls, 1 (5%) of 20; TFPILOW DOSE, 3 (25%) of 12 (P = .14 vs. control); TFPIHIGH DOSE, 7 (58.3%) of 12 (P = .002 vs. control); TFPIHIGH DOSE, 4 (30.8%) of 13 (P = .04). Mean survival times for the 4 study groups were as follows: vehicle controls, 27 h; TFPILOW DOSE, 68 h; TFPIHIGH DOSE, 109 h; TFPIHIGH DOSE, 93 h. Survival was significantly prolonged com-
pared with that in controls for all 3 TFPI-treated groups (TFPI_{LOW \ DOSE}, P = .04; TFPI_{MED \ DOSE}, P = .0001; TFPI_{HIGH \ DOSE}, P = .0002). Although survival times varied among the 3 TFPI-treated groups, none of the differences achieved statistical significance.

Circulating WBC counts were similar in all groups at baseline (figure 4A). At 4 h, WBC counts decreased significantly with respect to the 0-h value in all groups. However, at 10 and 16 h, WBC counts tended to return toward normal in all 3 TFPI-treated groups, whereas WBC counts continued to decrease in vehicle-treated controls. Platelet counts were similar among groups at baseline but decreased significantly over time in the control group (figure 1B). In contrast, platelet counts remained constant over time in septic rabbits treated with TFPI and were significantly higher than those in time-matched controls at 10 and 16 h.

Table 2 presents mean arterial pressure (MAP) and arterial partial pressure of oxygen (PaO_2) in control and TFPI-treated rabbits with bacterial peritonitis.

<table>
<thead>
<tr>
<th>Parameter, group</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaO_2 (mm Hg)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>109 ± 3</td>
</tr>
<tr>
<td>TFPI_{LOW \ DOSE}</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>TFPI_{MED \ DOSE}</td>
<td>111 ± 4</td>
</tr>
<tr>
<td>TFPI_{HIGH \ DOSE}</td>
<td>111 ± 3</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>75 ± 3</td>
</tr>
<tr>
<td>TFPI_{LOW \ DOSE}</td>
<td>71 ± 6</td>
</tr>
<tr>
<td>TFPI_{MED \ DOSE}</td>
<td>74 ± 4</td>
</tr>
<tr>
<td>TFPI_{HIGH \ DOSE}</td>
<td>79 ± 2</td>
</tr>
</tbody>
</table>

* P < .05 vs. time-matched value in control group (analysis of variance and Student-Newman-Keuls test).

Table 4 presents mean arterial blood pressure and partial pressure of oxygen in arterial blood (PaO_2) for control rabbits and rabbits treated with TFPI. Results obtained at 0 h are not presented (although the measurements were obtained), because the rabbits were deeply anesthetized at that time point and blood pressure and PaO_2 were low in all animals on that basis. Whereas blood pressure tended to increase over time in TFPI-treated rabbits, the opposite was true in controls. Compared with values in controls, mean arterial pressure values were significantly higher at 10 and 16 h in the TFPI_{HIGH \ DOSE} and TFPI_{MED \ DOSE} groups but not in the TFPI_{LOW \ DOSE} group. By 4 h, PaO_2 values were normal and were similar in all 4 groups. However, at 10 and 16 h, PaO_2 values were significantly lower than those in any of the TFPI-treated groups.

Base excess was similar and positive in all groups at baseline (figure 5A). Base excess decreased in all groups over time, although severe metabolic acidosis was evident from 0 to 16 h only in vehicle-treated controls. Values of base excess at 10 and 16 h were significantly lower in controls than in any of the TFPI-treated groups. Plasma bicarbonate concentration decreased in all groups, but the magnitude of this change was significantly greater in the control group than in any of the groups receiving an infusion of TFPI (figure 5B). Although arterial pH was similar and normal in all groups at baseline, over the next 16 h, acidemia developed in controls but not.
Figure 5. Effect of treatment with TFPI (3 different dosing regimens) or vehicle on arterial base excess (A), bicarbonate concentration (B), pH (C), and lactate concentration (D) in rabbits with gram-negative peritonitis. Sepsis was induced at 0 h and treatment was initiated at 4 h. Results are means ± SEs. * P < .05 vs. time-matched value in vehicle-treated group; † P < .05 versus 0-h value in same group (2-way analysis of variance for repeated measures and Student-Newman-Keuls test). Groups are as follows: □, TFPI_LOW DOSE; ○, TFPI_MID DOSE; ■, TFPI_HIGH DOSE; ○, control.

in the groups treated with TFPI (figure 5C). Blood lactate concentration was 1.2 ± 0.2 mM at 0 h in the control group and increased significantly, to 3.8 ± 0.4 mM, at 16 h (figure 5D). In contrast, blood lactate concentration did not change significantly over time in any of the TFPI-treated groups. At 10 and 16 h, blood lactate concentrations were significantly lower in all of the TFPI-treated groups than in the control group.

A number of parameters related to blood coagulation were measured. However, because a number of specimens were lost when a freezer thawed during a power outage, sample sizes were too small for detailed group-by-group analyses of data from all 4 treatment groups. Accordingly, data from all animals at 0 and 4 h (i.e., before and after the onset of sepsis but before the institution of any therapeutic interventions) have been pooled. In addition, for other analyses (table 3), data for the 3 TFPI-treated groups have been pooled for comparison with data from the vehicle-treated group. For all rabbits, prothrombin time increased slightly, from 7.3 ± 0.3 s at 0 h to 7.9 ± 0.3 s at 4 h (n = 40; P = .08). Similarly, partial thromboplastin time increased slightly, from 59.3 ± 12.0 s at 0 h to 63.4 ± 11.0 s at 4 h (n = 39; P = .21). Circulating fibrinogen levels decreased from 283 ± 30 mg/dL at 0 h to 183 ± 26 mg/dL at 4 h (n = 40; P = .08). During the interval from 0 to 4 h, circulating levels of factor V activity decreased substantially, from 1842 ± 125 to 468 ± 43 percentage activity units (n =
### Table 3. Blood coagulation parameters in control and TFPI-treated rabbits with gram-negative sepsis.

<table>
<thead>
<tr>
<th>Parameter, group</th>
<th>Time (h)</th>
<th>0</th>
<th>10</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s) Control</td>
<td>7.5 ± 0.5</td>
<td>7.6 ± 0.5</td>
<td>8.1 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>TFPI</td>
<td>7.8 ± 0.4</td>
<td>8.5 ± 0.4</td>
<td>7.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>PTT (s) Control</td>
<td>32.8 ± 6.1</td>
<td>36.5 ± 10.1</td>
<td>58.4 ± 30.1</td>
<td></td>
</tr>
<tr>
<td>TFPI</td>
<td>37.2 ± 4.1</td>
<td>31.4 ± 4.1</td>
<td>27.6 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen (mg/dL) Control</td>
<td>232 ± 29</td>
<td>265 ± 27</td>
<td>212 ± 41</td>
<td></td>
</tr>
<tr>
<td>TFPI</td>
<td>248 ± 37</td>
<td>432 ± 64</td>
<td>443 ± 58</td>
<td></td>
</tr>
<tr>
<td>Factor V (% activity) Control</td>
<td>4280 ± 1850</td>
<td>4130 ± 1920</td>
<td>2560 ± 1200</td>
<td></td>
</tr>
<tr>
<td>TFPI</td>
<td>2030 ± 1050</td>
<td>1040 ± 570</td>
<td>1390 ± 870</td>
<td></td>
</tr>
<tr>
<td>Factor X (% activity) Control</td>
<td>316 ± 43</td>
<td>278 ± 49</td>
<td>225 ± 38</td>
<td></td>
</tr>
<tr>
<td>TFPI</td>
<td>387 ± 74</td>
<td>435 ± 38</td>
<td>580 ± 70</td>
<td></td>
</tr>
<tr>
<td>Factor VII (% activity) Control</td>
<td>603 ± 151</td>
<td>519 ± 183</td>
<td>375 ± 138</td>
<td></td>
</tr>
<tr>
<td>TFPI</td>
<td>413 ± 65</td>
<td>378 ± 44</td>
<td>557 ± 102</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. Control, n = 3; TFPI, n = 8. Results shown are pooled data from all TFPI-treated groups. PT, prothrombin time; PTT, partial thromboplastin time. For factor X and fibrinogen, P for group × time effect by two-way repeated-measures analysis of variance was .02 and .07, respectively. For all other coagulation factors or parameters, P was not significant.

24; P = .03), and levels of factor VII activity decreased from 453 ± 78 to 320 ± 54 percentage activity units (n = 24; P = .07). Circulating levels of factor X increased from 482 ± 72 to 597 ± 88 percentage activity units (n = 40; P = .30). As shown in table 3, treatment with TFPI resulted in higher fibrinogen and factor X levels over the first 16 h of observation. Values for prothrombin time, partial thromboplastin time, factor V, and factor VII were similar between groups.

At necropsy, the lungs of nonsurvivors were grossly edematous, and the airways were filled with pink, frothy fluid. The kidneys were hemorrhagic and congested. The liver typically contained abscesses that grew out *E. coli*. Abscesses were present throughout the peritoneal cavity. In contrast, 7-day survivors showed evidence of minimal pulmonary congestion. Small scattered abscesses were often present in the peritoneal cavity, but other solid organs (adrenals, liver, spleen) were grossly normal.

### Discussion

Three lines of evidence support the notion that tissue factor–mediated events are important in the pathogenesis of sepsis. First, injection of human volunteers or experimental animals with lipopolysaccharide or tumor necrosis factor-α results in the generation of thrombin despite normal plasma levels of markers of intrinsic pathway activation (e.g., factor XIIa–C1 inhibitor complexes, kallikrein–C1 inhibitor complexes, and factor IX activation peptide) [28, 29]. Second, meningococcal sepsis in patients has been associated with increased tissue factor expression on peripheral blood monocytes [30]. Third, various antagonists of the extrinsic pathway of coagulation have been shown to have beneficial effects in animal models of DIC and gram-negative sepsis. For example, both tissue factor–neutralizing antibodies [31, 32] and factor VII/VIIa–neutralizing antibodies [33] have been shown to improve survival in animal models of septic shock. In addition, treatment with TFPI has been shown to ameliorate glomerular fibrin deposition in rabbits challenged with lipopolysaccharide [34] and improve survival in baboons infused with viable *E. coli* [24, 25].

The results in the present study confirm and extend the observations that inhibition of the extrinsic pathway of coagulation is beneficial in animal models of gram-negative sepsis. However, this is the first report to show that treatment with recombinant TFPI significantly improves survival time and long-term survival in rabbits with gram-negative peritonitis. TFPI was used as an adjuvant to therapy with conventional antimicrobial chemotherapy. The beneficial effect of treatment with TFPI was evident even when therapy was delayed as long as 4–6 h after the onset of infection (i.e., after unambiguous signs of sepsis were already present). In addition to improving survival, treatment with TFPI also had beneficial effects on a number of different physiologic parameters, including arterial blood pressure, arterial oxygenation, and peripheral blood platelet and leukocyte counts. While dosing of TFPI extended over a 10-fold range, only a 3-fold difference in circulating TFPI concentrations was observed between the highest and lowest doses of the compound studied (data not shown).

Since circulating concentrations of TFPI range from considerably less than normal to about twice normal in many cases of sepsis [20, 21], it is reasonable to wonder why administration of exogenous TFPI has been found to improve survival in two different animal models of sepsis. Unfortunately, presently available data are insufficient to answer this question definitively. In a previously published study, treatment with recombinant TFPI was shown to improve survival when infused in septic bunnies at a dosage designed to achieve a circulating TFPI concentration of 2000 ng/mL (i.e., ~20 times greater than normal) [24]. However, preliminary data from ongoing studies in our laboratory suggest that survival in septic rabbits is associated with circulating TFPI levels in the 300–1000 ng/mL range (i.e., ~3- to 10-fold greater than normal [unpublished data]). Accordingly, relatively small increases in circulating TFPI levels may be sufficient to provide protection against potentially lethal consequences of gram-negative infection. Furthermore, TFPI exists in vivo in three separate compartments: sequestered in platelets, bound to endothelial cells, and circulating in plasma [19]. It is possible, therefore, that measurements of circulating TFPI provide an incomplete profile of the functional availability of TFPI as an inhibitor of
DIC. Finally, recent in vitro studies indicate that recombinant TFPI binds to lipopolysaccharide and thereby blocks cellular responses to endotoxin by preventing the interaction of lipopolysaccharide with its cellular receptor (CD14) [35]. Thus, in gram-negative sepsis, exogenous TFPI may improve survival not only by inhibiting coagulation but also by neutralizing endotoxin.

The rabbit sepsis model used for these studies is characterized by the following features: Survival is the primary end point; sepsis is caused by a true infection (rather than the injection of purified endotoxin or some other microbial product); sepsis is induced by a relatively small initial inoculum (i.e., the microbes proliferate in vivo, leading ultimately to a potentially lethal burden of bacteria); sepsis is associated with a defined focus of infection (rather than caused by an intravenous infusion of viable microbes); the magnitude of the septic challenge is under tight experimental control; the experimental agent (i.e., TFPI) is evaluated as adjunct to conventional antimicrobial chemotherapy; treatment with the experimental agent is instituted after the onset of clinical signs of sepsis; and sepsis is caused by a clinically relevant pathogenic organism. In this model, sepsis is induced by implanting in the peritoneal cavity a known quantity of viable organisms suspended in a mixture containing hemoglobin and mucin. Both hemoglobin [36–39] and mucin [37, 40, 41] are well-known “adjuvants” capable of markedly diminishing the size of the initial inoculum of organisms required to cause mortality in experimental infections. These adjuvants are thought to promote the proliferation of the bacteria in vivo [39] or interfere with the host’s defenses against the infectious challenge [36, 42, 43] (or both).

Treatment with the experimental agent (or the appropriate control substance) was started at 4 h. By this time point, the animals showed obvious clinical manifestations of systemic inflammation (conjunctival injection, lethargy) as well as laboratory evidence of sepsis (leukopenia). As would be the case in the clinical setting, treatment with TFPI (or the control substance) was started at the same time as the initiation of therapy with an appropriate antibiotic. Thus, TFPI was evaluated in the context of standard antimicrobial chemotherapy.

As recently pointed out by Cross et al. [26], sepsis models should use pathogenic microorganisms endowed with features (e.g., presence of a polysaccharide capsule) that interfere with the opsonophagocytic mechanisms of the host. It is noteworthy in this regard that of the >170 identified O-specific serotypes of E. coli, only ~12 are commonly identified in patients with bacteremias caused by the microbial species [26]. Interestingly, many of the strains of E. coli that have been used commonly in laboratory models of sepsis lack the virulence factors associated with ability to survive and proliferate in vivo [26]. The strain of E. coli used for the present study, O18:K1, is commonly responsible for bloodstream infections in humans and is highly pathogenic [26].

Because the rabbit has been widely used for studies of lipopolysaccharide-induced inflammation and coagulation, the structural and functional similarities and differences between the human and lapine forms of TFPI have been investigated. Both forms of the protein consist of three Kunitz domains as well as negatively charged head (amino-terminus) and positively charged tail (carboxyl-terminus) regions. Overall, there is a moderate degree of sequence homology between the human and lapine proteins, although some important differences have been reported [44]. For example, lapine TFPI has a glycosylation site at residue 245 (asparagine), which is absent from the human protein [44]. This observation is consistent with data showing that rabbit TFPI is more extensively glycosylated than is human TFPI [45]. Whereas human TFPI binds to lipoproteins, the lapine form does not [45]. Human recombinant TFPI expressed in E. coli (i.e., the form of the protein used in the present study) has essentially the same biologic activity in normal rabbit plasma as in normal human plasma (unpublished data).

The present studies, while clearly supporting the view that TFPI has considerable potential as an adjuvant agent for the treatment of gram-negative sepsis in patients, provide little information regarding the mechanism(s) underlying the agent’s therapeutic effects. Treatment with TFPI ameliorated the consumption of fibrinogen and factor X induced by sepsis in rabbits, suggesting that inhibition of DIC may be a factor contributing to the salutary effects of the agent. Lactate levels were lower and base excess levels were higher in TFPI-treated compared with vehicle-treated rabbits, suggesting that the apparent increases in anaerobic metabolism induced by sepsis were ameliorated by the recombinant protein. These findings might be interpreted as being consistent with prevention of DIC-induced derangements in microvascular perfusion in TFPI-treated rabbits. However, lactate production can increase in sepsis for reasons unrelated to the adequacy of tissue perfusion [46], and, therefore, the underlying mechanism(s) responsible for the lower blood lactate levels in TFPI-treated compared with control septic rabbits remains to be established.

Although experiment 3 was designed as a dose-response study, we were unable to detect clear dose-dependent effects on survival or various physiologic parameters. While the middle dose of TFPI (0.5 mg/kg bolus plus 5 μg/kg/min for 24 h) seemed to be the most effective of the three doses studied, differences among groups were too small or too inconsistent to achieve statistical significance. At present, we can offer no adequate explanation for the apparent lack of a dose-response relationship. Two possibilities, however, must be considered. First, even the lowest dose of TFPI used may have been greater than the level providing maximal benefit with respect to survival in rabbits with gram-negative peritonitis. Second, higher doses of TFPI may have been associated with toxicity (manifested as decreased survival), which masked any improvement in the therapeutic action of the agent.

In summary, we showed herein that recombinant TFPI significantly improves survival and other physiologic parameters in a very stringent and clinically relevant animal model of focal...
infection leading to lethal gram-negative sepsis. TFPI warrants further development as an adjuvant treatment for gram-negative sepsis in humans.

References


