Elevated levels of circulating procoagulants like tissue factor may increase the risk of systemic coagulation activation, thrombin generation, and consumptive coagulopathy. I measured procoagulant activity in plasma by using a clot-based assay that incorporated normal plasma to replace missing factors, corn trypsin inhibitor to block contact activation, factor VIIa to improve sensitivity to tissue factor activity, and anti–tissue factor antibodies to measure tissue factor–specific activity. Procoagulant activity was evaluated in 58 trauma patients. Trauma patients without coagulopathy (n = 50) showed 5-fold higher procoagulant activity than did control subjects (P < .001), whereas trauma patients with coagulopathy (n = 8) showed 10-fold higher activity than control subjects (P < .001) and 2-fold higher activity than trauma patients without coagulopathy (P = .03).

In control subjects, tissue factor activity was below the detection limit of the assay. Tissue factor activity was 3- to 4-fold higher in trauma patients with coagulopathy vs patients without coagulopathy (P = .002). Trauma patients with coagulopathy have increased circulating tissue factor activity.

In a number of clinical disorders, including shock, trauma, and sepsis, systemic coagulation activation can occur, leading to varying degrees of coagulation factor consumption and vascular fibrin deposition. Systemic exposure or release into the vascular system of substances that activate the coagulation system (procoagulants) may have a role in consumptive coagulopathies. Normal blood from healthy patients contains little procoagulant activity. If contact activation is blocked in normal blood, little if any thrombin generation occurs. In disease states, though, blood may contain several different forms of procoagulant that can help initiate thrombin generation, including soluble, cell, and microparticle-bound tissue factor; cancer-derived procoagulants; procoagulant phospholipids; activated factor X1a; and others. Acute coagulopathy of trauma (ACT) has been described that can have adverse effects on patient outcome. This trauma coagulopathy seems to arise from a combination of tissue trauma and shock, exacerbated by hemodilution, hypothermia, acidemia, and inflammation. ACT is associated with activation of the coagulation and fibrinolytic systems. Trauma patients are reported to have increased levels of circulating procoagulant phospholipids, tissue factor–bearing microparticles, and activated platelets and monocytes. It has been hypothesized that these circulating procoagulants may enhance the coagulopathy in severely injured patients, but this has not been studied directly. The purpose of this study was to develop a relatively simple, fast assay for measuring procoagulant activity in plasma and to use this assay to evaluate trauma patients with and without coagulopathy.
Materials and Methods

Human Subjects

The study was approved by the University of Washington Human Subjects Review Committee (Seattle). Blood samples were anticoagulated with 0.105 mol/L citrate. For the interference and recovery studies, samples were obtained from healthy control subjects, patients receiving warfarin (international normalized ratio, mean, 3.8; range, 2.5-11.1), patients receiving unfractionated heparin (activated partial thromboplastin time [aPTT], mean, 91 seconds; range, 60-200 seconds), and patients with lupus inhibitors. Positive lupus inhibitor samples had the following: (1) a prolonged aPTT, (2) incomplete correction after mixing with citrate-anticoagulated pooled normal plasma, (3) complete correction after addition of hexagonal phase phospholipids, and (4) no evidence of other coagulation factor inhibitors (normal aPTT after phospholipid addition, normal prothrombin time (PT), and normal thrombin time).

Samples were obtained from 58 trauma patients within 1 hour of arrival at the hospital. The International Society on Thrombosis and Haemostasis (ISTH) overt disseminated intravascular coagulation (DIC) score was determined for each subject based on platelet count (Abbott Diagnostics 3700 cell counter, Abbott Park, IL), fibrinogen (Diagnostica Stago Fibrinogen reagent and the STA-R instrument, Diagnostica Stago, Parsippany, NJ), D-dimer (fibrinogen equivalent units measured using Diagnostica Stago D-dimer latex immunoassay on the STA-R instrument), and PT (Diagnostica Stago Neoplastin CI+ reagent, international sensitivity index = 1.27, on the STA-R instrument).

The ISTH scoring system for overt DIC assumes a clinical diagnosis consistent with possible DIC (in this case, major trauma), then uses clinical testing results for platelet count, PT, fibrinogen, and fibrin-related markers (D-dimer) to assess the probability of overt DIC. Platelet counts less than 100 × 10^9/L (100 × 10^3/μL) scored 1 point and less than 50 × 10^9/L (50 × 10^3/μL), 2 points. Scoring for D-dimer was defined in agreement with a previously reported validation of ISTH DIC scoring using the STA D-Dimer LIATEST immunoturbidimetric method (Diagnostica Stago) as follows: “no increase” if less than 0.6 mg/L (1.8 nmol/L), 0 points; “moderate increase” if 0.6 to 8.2 mg/L (1.8-24.1 nmol/L), 2 points; and “strong increase” if greater than 8.2 mg/L (24.1 nmol/L), 3 points. PTs prolonged by more than 3 seconds (>18.5 seconds) scored 1 point, and those prolonged by more than 6 seconds (>21.5 seconds) scored 2 points. Fibrinogen levels of less than 1 g/L scored 1 point. Prior studies and a recent summary have found that an ISTH overt DIC score of 5 or more was most indicative of DIC-like coagulopathy. Based on this finding, I used an overt DIC score of 5 or higher as evidence of coagulopathy.

Materials

Inhibitory goat antihuman tissue factor IgG was obtained from American Diagnostica, Greenwich, CT. Lipidated recombinant human tissue factor (RecombiPlasTin) was obtained from Instrumentation Lab, Bedford, MA. Corn trypsin inhibitor and plasma-derived human activated factor VII were obtained from EMD Biosciences, Gibbstown, NJ. 1-α-phosphatidylcholine and 1-α-phosphatidylserine were obtained from Avanti Polar Lipids, Alabaster, AL. Phospholipid vesicles composed of 0.8 mmol/L phosphatidylcholine and 0.2 mmol/L phosphatidylserine in 20 mmol/L HEPES, 100 mmol/L sodium chloride, and 0.2 g/L sodium azide, pH 7.5, were prepared by drying the phospholipids under dry nitrogen, then strong vacuum, followed by suspension in HEPES buffer and sonication. Pooled normal plasma anticoagulated with 0.105 mmol/L citrate was obtained from Precision Biologics, Dartmouth, Canada.

Procoagulant Activity Assay

The clot-based procoagulant activity assay was based on prior methods. Exposure of recalcified citrate plasma to artificial surfaces may result in coagulation activation through the contact (kallikrein/kinin) system forming factor XIIa. To block contact activation, corn trypsin inhibitor was added. Human factor VIIa was added to improve sensitivity to tissue factor activity. Because the final clotting time was potentially dependent on the concentration of other clotting factors in the plasma, the sample plasma was mixed 1 to 1 with pooled normal plasma containing undetectable procoagulant activity to replace any other coagulation factors that may be low in the sample plasma. In the final assay, 20 μL of citrate plasma was added to 20 μL of pooled normal plasma and 60 μL of assay reagent containing 250 mg/L corn trypsin inhibitor, 62.5 μg/L human activated factor VII, 50 μmol/L phosphatidylcholine plus 12.5 mmol/L phosphatidylserine vesicles in 20 μmol/L HEPES, 100 mmol/L sodium chloride, 0.2 g/L sodium azide, and 1 g/L bovine serum albumin, pH 7.5. The mixture was incubated for 2 minutes at 37°C followed by recalcification with 50 μL of 25 mmol/L calcium chloride. Clotting times were determined by using a STA 4 semiautomated hemostasis analyzer (Diagnostica Stago). The maximum clotting time for this instrument is 999 seconds.

Lipidated recombinant human tissue factor was used as a typical procoagulant to standardize the activity assay. I defined 1 arbitrary unit (AU) of procoagulant activity as 1 ng of RecombiPlasTin lipidated recombinant human tissue factor. The procoagulant assay can potentially detect other procoagulants in addition to tissue factor. To determine how much of the procoagulant activity was due to tissue factor–like activity, the clotting time was measured in the presence and absence of 100 mg/L of anti–tissue factor antibody added to the reaction mixture 10 minutes before recalcification.
Chandler / Procoagulant Activity in Trauma

Statistics
Results are given as the mean ± SD unless otherwise indicated. Standard curves were based on linear regression of log-transformed variables. Differences between the trauma with and without coagulopathy groups were compared by using the Mann-Whitney test. Differences were considered significant when the P value was less than .05.

Results

Development of the Procoagulant Activity Assay
In vitro contact activation during the procoagulant activity assay caused variably shorter clotting times, resulting in falsely increased estimates of in vivo procoagulant activity. In the absence of contact system inhibition, the detection limit for procoagulant activity was 5 to 10 AU/L. Corn trypsin inhibitor was added to block factor XIIa formed by contact activation Figure 1. In pooled normal plasma, addition of at least 50 mg/L of corn trypsin inhibitor resulted in a clotting time of 999 seconds, the maximum for the instrument used. When corn trypsin inhibitor was added to pooled normal plasma spiked with tissue factor, maximum inhibition of contact activation occurred between 50 and 100 mg/L corn trypsin inhibitor. I selected 100 mg/L of corn trypsin inhibitor as the concentration in the final recalcified plasma mix. With corn trypsin inhibitor present, the detection limit for procoagulant activity was less than 1 AU/L.

Next, I evaluated the effect of adding recombinant human activated factor VII to the assay to improve the sensitivity of the assay to active tissue factor Figure 2. In pooled normal plasma with no evidence of detectable tissue factor activity and corn trypsin inhibitor added, addition of up to 50 μg/L of human activated factor VII had no effect on clotting times, indicating that the factor VIIa itself did not initiate clotting. When tissue factor was added to pooled normal plasma, the detection limit of the assay improved from between 3 and 10 AU/mL in the absence of added activated factor VII to less than 1 AU/mL with addition of 25 μg/L of human activated factor VII. Addition of 50 μg/L of activated factor VII did not improve the assay detection limit; therefore, I selected 25 μg/L as the concentration in the final recalcified plasma mix.

With corn trypsin inhibitor and activated factor VII, pooled normal plasma typically had a clotting time near the maximum clotting time of the instrument, 999 seconds. The procoagulant activity assay produced a linear response when the log of the clotting time was plotted against the log of the recombinant human tissue factor concentration diluted in pooled normal plasma Figure 3.21,22 The detection limit, based on the apparent tissue factor activity at 999 seconds from the calibration curve for each run, was 0.6 ± 0.2 AU/L (n = 16).

I evaluated the effect of 100 mg/L anti-tissue factor antibody on procoagulant activity in pooled normal plasma, pooled normal plasma plus human recombinant tissue factor, and patient samples Table 1. Pooled normal plasma had a clotting time of 999 seconds before and after addition of anti-tissue factor antibody, indicating the antibody had no effect on the assay when tissue factor was not present in detectable amounts. In pooled normal plasma containing 500 ng/L (500 AU/L) human recombinant tissue factor, addition of anti-tissue factor antibody inhibited more than 99% of the exogenous tissue factor added, resulting in a clotting time of 999 seconds.
Addition of anti–tissue factor antibody to 2 patient samples inhibited the majority of procoagulant activity in the samples. The purpose of the assay was to detect substances in plasma that would activate the coagulation system, procoagulants like tissue factor, not the level of coagulation factors. I anticipated that low coagulation factor levels might affect assay results; therefore, as part of the assay design, patient samples were mixed 1:1 with pooled normal plasma (clotting time typically near 999 seconds) before running the assay. Other factors, such as heparin or lupus inhibitors that could inhibit clotting in samples, might also lead to long clotting times and falsely low estimates of procoagulant activity; therefore, I measured the recovery of human recombinant tissue factor added to plasma samples from healthy subjects, patients receiving warfarin, patients receiving heparin, and patients with lupus inhibitors Table 21. Recovery was determined by measuring procoagulant activity before and after addition of 20 ng/L human recombinant tissue factor.

The average recovery of added procoagulant activity in 5 healthy subjects was 99%. In patients receiving warfarin, average recovery was 103%, indicating that low factor levels due to warfarin therapy did not affect assay results. Samples from patients receiving heparin gave prolonged clotting times and lower recovery of added procoagulant activity (average, 21% recovery), presumably due to inhibition of clotting in the sample by heparin. If heparin was removed using epichlorohydrin triethanolamine cellulose before measuring procoagulant activity, recovery of recombinant human tissue factor added before heparin removal increased to 103%, similar to the recovery seen in normal plasma. Samples that contained lupus inhibitors also showed lower recovery, on average 32% of the expected level.

Some prior studies have added phosphatidylcholine-phosphatidylserine vesicles to clot-based tissue factor activity assays, whereas others have not. Because lupus inhibitors may be neutralized in some cases by addition of exogenous anionic phospholipids, I compared recovery in normal plasma and plasma with lupus inhibitors after adding 25 μmol/L phospholipid vesicles to the clot-based activity assay. Addition of phospholipid vesicles had no effect on recovery from normal plasma but improved recovery from most plasma samples with lupus inhibitors. Of 7 plasma samples with lupus inhibitors tested using the clot-based reagent containing phospholipid vesicles, 4 had average recovery of near or more than 100%, similar to recovery in other samples. In the remaining 3 samples, recovery was only 15% to 62%. This finding indicates that lupus inhibitors can interfere with measurement of procoagulant activity but that addition of phospholipid vesicles at a final assay concentration of 25 μmol/L can reduce lupus anticoagulant interference.

In the final procoagulant activity assay, the final reaction concentrations were 100 mg/L corn trypsin inhibitor, 25 μg/L human recombinant activated factor VII, and 25 μmol/L phospholipid. With this assay, the within-run imprecision for the procoagulant activity assay was 25 ± 2 AU/L (coefficient of variation, 9%), whereas the between-run imprecision for the procoagulant assay was 30 ± 4 AU/L (coefficient of variation, 10%).

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Mean ± SD Procoagulant Activity Recovery (%)</th>
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<tbody>
<tr>
<td>Normal plasma</td>
<td>99 ± 15</td>
</tr>
<tr>
<td>Warfarin (INR &gt; 2)</td>
<td>103 ± 22</td>
</tr>
<tr>
<td>Heparin</td>
<td>21 ± 26</td>
</tr>
<tr>
<td>Lupus inhibitor</td>
<td>32 ± 23</td>
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*To determine procoagulant activity recovery from plasma samples with a potential source of interference (warfarin, positive lupus inhibitor, or therapeutic heparin), procoagulant activity was measured before and after addition of 20 arbitrary units per liter of recombinant human tissue factor (n = 5 for each sample type).
14%). The reference range for procoagulant activity based on plasma samples from 10 healthy men (39 ± 15 years) and 10 healthy women (38 ± 12 years) was 1.4 ± 0.5 AU/L. Tissue factor activity was below the detection limit of the assay.

Procoagulant Activity in Trauma Patients

Samples from 58 trauma patients (47 men and 11 women; age, 46 ± 17 years) were evaluated, including samples from 47 patients with blunt trauma (motor vehicle crashes and falls) and 11 with penetrating injuries (gunshot wounds and stabbing). Of the 58 trauma patients evaluated, Table 3, 8 showed evidence of coagulopathy (7 men and 1 woman; DIC score, 5.8 ± 0.5), whereas 50 did not (DIC score, 2.4 ± 1.1). Procoagulant activity was higher than the maximum level seen in control subjects (2.9 AU/L) in 56 of 58 trauma patients, 49 of 50 without coagulopathy, and 7 of 8 with coagulopathy (Figure 4). Trauma patients with coagulopathy had 10-fold higher levels of procoagulant activity than did control subjects (P < .001) and 2-fold higher than trauma patients without coagulopathy (P = .03).

Contribution of Tissue Factor Activity

In plasma from healthy subjects, tissue factor activity was below the detection limit of the assay. In trauma patients without coagulopathy, 47% ± 18% of procoagulant activity was due to tissue factor activity (Figure 5). In trauma patients with coagulopathy, 79% ± 13% of procoagulant activity was due to tissue factor activity, which was 3- to 4-fold higher than in trauma patients without coagulopathy (P = .002).

Discussion

I developed a plasma procoagulant activity assay based on modification of prior methods. An early tissue factor activity assay added tissue factor–containing samples directly to normal plasma followed by recalcification. This assay had a reported detection limit of about 100 ng/L of active tissue factor (~2 pmol/L). Addition of corn trypsin inhibitor to block contact activation in the sample improved the sensitivity to tissue factor, resulting in detection limits for tissue factor activity of 1 to 2 ng/L (~20–40 fmol/L). To measure tissue factor activity specifically, procoagulant activity was measured in the presence and absence of inhibitory anti–tissue factor antibody. To eliminate the effect of low coagulation factor levels on results, I combined plasma samples 1 to 1 with pooled normal plasma containing low background procoagulant activity. In addition to normal plasma and corn trypsin inhibitor, I added phospholipid to help block lupus inhibitors and activated factor VII, which had no effect on normal plasma but improved the detection limit when tissue factor activity was present. This final assay could be completed in 30 to 60 minutes, making it potentially useful for evaluation of patients suspected of having coagulation activation and factor consumption.

<table>
<thead>
<tr>
<th>Hemostasis Data for Trauma Patients*</th>
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<tbody>
<tr>
<td><strong>Without Coagulopathy</strong></td>
</tr>
<tr>
<td>Prothrombin time (s)</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
</tr>
<tr>
<td>Platelet count (× 10^9/L)</td>
</tr>
<tr>
<td>D-dimer (nmol/L)</td>
</tr>
<tr>
<td>Procoagulant activity (AU/L)</td>
</tr>
<tr>
<td>Tissue factor–like activity (AU/L)</td>
</tr>
</tbody>
</table>

AU/L, arbitrary units per liter.
* Data are given as mean ± SD. Values are given in Système International units; conversions to conventional units are as follows: D-dimer (g/L), divide by 5.476; fibrinogen (mg/ dL), divide by 0.01; platelet count (× 10^9/L), divide by 1.0.
† Evidence of coagulopathy based on an International Society on Thrombosis and Haemostasis overt disseminated intravascular coagulation score of ≥5.
and without coagulopathy about as well as other factors, like
sue factor activity levels discriminated trauma patients with
overlap between groups (Figures 4 and 5). On average, tis-
patients with and without coagulopathy shows considerable
blood.6,22 Review of the scattergrams comparing trauma
activity made up only part of the procoagulant activity in
prior studies for which reports stated that tissue factor
coagulopathy. Not all procoagulant activity was due to tis-
was 3- to 4-fold higher than that of trauma patients without
coagulopathy. Most predictive of coagulopathy, though, was
the level of tissue factor activity in plasma. Trauma patients
coagulopathy on average had tissue factor activity that
showed little detectable procoagulant or tissue factor activ-
In contrast, the majority of trauma patients had elevated
procoagulant activity in plasma, with the highest levels in
trauma patients with coagulopathy, 10-fold more than in con-
rolled subjects and 2-fold more than in trauma patients without
coagulopathy. Most predictive of coagulopathy, though, was
this may lead to activation of the coagulation and fibrinolytic
Thus, circulating plasma tissue factor activity is
only one part of a process that may lead to coagulopathy.
Trauma results in a variety of alterations to the hemostat-
ic system that can lead to an increased risk of bleeding soon
after injury7 and an increased risk of thrombosis later.23 Some
severely injured patients have normal PT, fibrinogen, and
platelet count values on arrival, but these hemostatic factors
are rapidly depleted once warming and resuscitation begin.
Further study of tissue factor activity over time in comparison
with changes in hemostatic factors is needed to determine
whether procoagulant and tissue factor activity levels are
associated with risk of early consumptive coagulopathy and/
or late risk of thrombosis.

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Figure 5: Scattergram of tissue factor activity in trauma
patients without coagulopathy and trauma patients with
coagulopathy. AU/L, arbitrary units per liter.

Dunbar and Chandler24 recently reported that trauma
patients with ACT showed dysregulated hemostasis charac-
terized by excessive non–wound-related thrombin generation
due to a combination systemic activation of coagulation
and reduced inhibitor levels, allowing systemic thrombin
generation to continue once started. It was hypothesized that
non–wound-related systemic thrombin generation in severe
trauma may occur in part due to circulating procoagulants.
As in prior studies,1,2,6,22,24 healthy subjects in this study
showed little detectable procoagulant or tissue factor activ-
ity. In contrast, the majority of trauma patients had elevated
procoagulant activity in plasma, with the highest levels in
trauma patients with coagulopathy, 10-fold more than in con-
rol subjects and 2-fold more than in trauma patients without
coagulopathy. Most predictive of coagulopathy, though, was
the level of tissue factor activity in plasma. Trauma patients
coagulopathy on average had tissue factor activity that
was 3- to 4-fold higher than that of trauma patients without
coagulopathy. Not all procoagulant activity was due to tis-
ue factor activity. In trauma patients without coagulopathy,
on average, only half of the procoagulant activity was due
to tissue factor activity. In trauma patients with coagul-
opathy, tissue factor activity made up approximately 80% of
total procoagulant activity. This finding is similar to those
in prior studies for which reports stated that tissue factor
activity made up only part of the procoagulant activity in
blood.6,22 Review of the scattergrams comparing trauma
patients with and without coagulopathy shows considerable
overlap between groups (Figures 4 and 5). On average,
tissue factor activity levels discriminated trauma patients with
and without coagulopathy about as well as other factors, like
PT, fibrinogen level, platelet count, and D-dimer level, used
in the ISTH DIC score (Table 3), but tissue factor activity
levels were not clearly better.

ACT is a complex process that seems to arise from a
combination of tissue trauma and shock, exacerbated by
hemodilution, hyperthermia, acidemia, and inflammation.29
Studies have shown that severe injury increases circulating
procoagulant phospholipids,13 tissue factor–bearing micropar-
ticles,14 and activated platelets and monocytes15-17 and that
this may lead to activation of the coagulation and fibrinolytic
systems.10-12 Thus, circulating plasma tissue factor activity
is one only part of a process that may lead to coagulopathy.

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