Cell-Free DNA Modulates Clot Structure and Impairs Fibrinolysis in Sepsis

Travis J. Gould, Trang T. Vu, Alan R. Stafford, Dhruba J. Dwivedi, Paul Y. Kim, Alison E. Fox-Robichaud, Jeffrey I. Weitz, Patricia C. Liaw

Objectives—Sepsis is characterized by systemic activation of inflammation and coagulation in response to infection. In sepsis, activated neutrophils extrude neutrophil extracellular traps composed of cell-free DNA (CFDNA) that not only trap pathogens but also provide a stimulus for clot formation. Although the effect of CFDNA on coagulation has been extensively studied, much less is known about the impact of CFDNA on fibrinolysis. To address this, we (1) investigated the relationship between CFDNA levels and fibrinolytic activity in sepsis and (2) determined the mechanisms by which CFDNA modulates fibrinolysis.

Approach and Results—Plasma was collected from healthy and septic individuals, and CFDNA was quantified. Clot lysis assays were performed in plasma and purified systems, and lysis times were determined by monitoring absorbance. Clot morphology was assessed using scanning electron microscopy. Clots formed in plasma from septic patients containing >5 µg/mL CFDNA were dense in structure and resistant to fibrinolysis, a phenomenon overcome by deoxyribonuclease addition. These effects were recapitulated in control plasma supplemented with CFDNA. In a purified system, CFDNA delayed fibrinolysis but did not alter tissue-type plasminogen activator–induced plasmin generation. Using surface plasmon resonance, CFDNA bound plasmin with a $K_d$ value of 4.2±0.3 µmol/L, and increasing concentrations of CFDNA impaired plasmin-mediated degradation of fibrin clots via the formation of a nonproductive ternary complex between plasmin, CFDNA, and fibrin.

Conclusions—Our studies suggest that the increased levels of CFDNA in sepsis impair fibrinolysis by inhibiting plasmin-mediated fibrin degradation, thereby identifying CFDNA as a potential therapeutic target for sepsis treatment.

Key Words: fibrinolysis ■ plasminogen activator inhibitor 1 ■ sepsis ■ tissue plasminogen activator

Sepsis is initiated by the release of microorganisms and microbial toxins into the circulation, although infection itself is rarely the cause of death in these patients.1–3 Rather, mortality in septic patients is attributed to irreversible organ failure from prolonged, uncontrolled activation of inflammatory and coagulation pathways within the microcirculation. Despite many recent advances in management and treatment, sepsis remains the leading cause of morbidity and mortality in noncoronary intensive care units in North America.4–7 Severe sepsis, defined as sepsis associated with at least 1 dysfunctional organ, affects ≈750,000 individuals in the United States annually.8,9 Sepsis-induced mortality remains high, ranging from 18% to 30%, and is further increased if disseminated intravascular coagulation occurs.5,8,10,11

Clinical management of patients with sepsis is challenging and largely limited to supportive therapies, which is in part related to a limited understanding of the underlying pathophysiology.12,11 Recently, cell-free DNA (CFDNA) has emerged as an important link between innate immunity, coagulation, and inflammation.14–16 Furthermore, we have previously demonstrated that the plasma levels of CFDNA have high discriminative power to predict intensive care unit mortality in patients with severe sepsis.7 Patients with higher plasma concentrations of CFDNA are more likely to face severe complications, such as organ dysfunction/failure, and death. This evidence suggests that CFDNA may not simply be an innocuous biomarker of severity but may itself exert pathological effects in sepsis.

CFDNA is released through various cellular processes, including apoptosis, necrosis, or by neutrophils, as a component of neutrophil extracellular traps (NETs).6,17–21 NETs are composed of extracellular DNA, histones, and neutrophil granular proteins. CFDNA, either in the presence or absence of histones, has been shown to modulate several procoagulant pathways. CFDNA circulates at low levels in health individuals (0.02–1.7 µg/mL),22,23 but elevated circulating CFDNA levels (0.1–5 µg/mL)24,25 have been detected in a variety of disease states, including sepsis. Elevated levels of CFDNA in septic
patients increase thrombin generation by activating the intrinsic pathway of blood coagulation, whereas DNA–histone complexes trigger platelet activation and aggregation by signaling through toll-like receptor-2 and toll-like receptor-4. In addition, recent evidence suggests that fibrin, along with von Willebrand factor and chromatin, form a colocalized network within the thrombus that provides a scaffold for localized coagulation activation coupled with platelet and red blood cell adhesion, thereby promoting thrombus formation.

Although the contributions of CFDNA to coagulation activation and thrombus formation have been well characterized, studies on the influence of CFDNA on the fibrinolytic system are limited. NETs have previously been shown to intercalate with fibrin to form a structural network that is resistant to lysis by tissue-type plasminogen activator (tPA) or degradation by DNase. Conversely, CFDNA has been shown to facilitate the recruitment of profibrinolytic enzymes, including tPA, urokinase plasminogen activator, plasminogen, and plasmin, and their endogenous inhibitors, plasminogen activator inhibitor-1 (PAI-1) and α2-antiplasmin. Thus, although CFDNA may augment fibrinolytic activity by enhancing fibrin-independent plasminogen activation, it may also suppress fibrinolysis by increasing the susceptibility of fibrinolytic enzymes to inhibition.

The levels of CFDNA are elevated in patients with severe sepsis. Consequently, samples from septic patients provide an opportunity to examine the influence of CFDNA on fibrinolysis in the plasma milieu. Therefore, in this study, we (1) measured fibrinolysis in plasma samples from septic patients and examined the influence of endogenous CFDNA on this process, (2) investigated the impact of CFDNA on the structure of plasma clots, and (3) defined the mechanisms by which CFDNA modulates fibrinolytic activity.

### Materials and Methods

The Study Design and Experimental Methods are described in detail in the online-only Data Supplement.

### Results

#### Elevated Levels of Endogenous CFDNA Impairs Fibrinolysis in Septic Patient Plasma

To study the effects of elevated endogenous CFDNA levels on fibrinolysis, plasma samples from 60 patients with severe sepsis and 10 healthy donors were examined. The baseline (day 1) characteristics of the 60 severe sepsis patients are shown in Table 1. CFDNA was quantified, and based on levels, plasma samples were arbitrarily categorized into low (0–4.9 µg/mL), intermediate (5–9.9 µg/mL), and high (>10 µg/mL) CFDNA subgroups. Plasma samples were supplemented with 1 nmol/L tPA, and clotting was initiated by addition of CaCl₂. Clots formed in plasma samples from healthy controls (n=10) and patients with low levels of endogenous CFDNA (n=20) underwent complete lysis. In contrast, of the clots formed in plasma samples containing intermediate or high levels of CFDNA, only 35% and 10%, respectively, underwent complete lysis during the 5-hour period of observation (Figure 1A). The delay in clot lysis correlated with decreased plasma levels of α2-microglobulin, consistent with reduced fibrin degradation (Table 2). No significant differences were observed between other potential mediators of fibrinolysis, including thrombin activatable fibrinolysis inhibitor or α2-antiplasmin (Table 2).

To determine the contribution of CFDNA to the reduced fibrinolysis, plasma samples were preincubated with 20 µg/mL DNase I. RNase and the PAI-1 inhibitory antibody MA-55F (which was first confirmed to be effective in inhibiting PAI-1 activity in a plasma environment; data not shown) were used as controls. Neither preincubation with RNase nor MA-55F affected clot lysis. In contrast, pretreatment with DNase partially restored fibrinolytic activity in samples containing intermediate and high levels of CFDNA (Figure 1A). In the absence of tPA, DNase alone exerted no fibrinolytic activity (data not shown). Collectively, these results suggest that the presence of elevated levels of CFDNA in septic patient plasmas impairs fibrinolytic activity.

### Evidence of Altered Structure of Clots Formed in Plasmas From Septic Patients With Elevated Levels of CFDNA

Turbidity, as determined by spectrophotometric absorbance, is indicative of fibrin clot structure: thick fibers and dense clot morphology results in an increase in final turbidity. Although maximum absorbance values in samples with low levels of CFDNA were similar to those in control samples, maximum absorbance values were 3-fold and 2-fold higher in samples with intermediate or high CFDNA levels, respectively (P<0.001 and P=0.004; Figure 1B). Addition of DNase but not RNase or MA-55F reduced the peak turbidity of the plasma samples containing intermediate or high CFDNA levels (Figure 1B). This suggests that the presence of elevated CFDNA levels correspond with altered clot structure composed of thicker fibers in a densely packed fibrin network.

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### Table 1. Baseline Characteristics of 60 Patients With Severe Sepsis

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y, mean±SEM (minimum, maximum)</td>
<td>61.33±2.2 (22, 93)</td>
</tr>
<tr>
<td>Sex, % women (no./total)</td>
<td>45 (27/60)</td>
</tr>
<tr>
<td>APACHE II score, mean±SE (minimum, maximum)</td>
<td>26.47±1.11 (7, 52)</td>
</tr>
<tr>
<td>MODS score, mean±SE (minimum, maximum)</td>
<td>7.25±0.47 (1, 17)</td>
</tr>
<tr>
<td>Positive cultures, % positive (no./total)</td>
<td>70 (42/60)</td>
</tr>
<tr>
<td>Infection type, % (no./total)</td>
<td>21.4 (9/42)</td>
</tr>
<tr>
<td>Gram positive</td>
<td>31 (13/42)</td>
</tr>
<tr>
<td>Fungal</td>
<td>16.7 (7/42)</td>
</tr>
<tr>
<td>Viral</td>
<td>28.6 (12/42)</td>
</tr>
<tr>
<td>Mixed</td>
<td>2.4 (1/42)</td>
</tr>
</tbody>
</table>

APACHE II indicates Acute Physiology and Chronic Health Evaluation II.
To investigate the effect of CFDNA on clot morphology, we used scanning electron microscopy to compare the clot structure of clots formed in plasma from septic patients with those generated in control plasma. Whereas clots formed in control plasmas were loosely packed and composed of thin fibrin fibers (Figure 1C; Table 3), those formed in plasma from septic patients were more tightly packed and composed of thicker individual fibrin fibers (Figure 1D and 1E; Table 3).

Interestingly, clots formed in plasmas containing the highest levels of CFDNA appeared to be less dense, although the individual fibers remained thicker (Figure 1F; Table 3).

To identify potential factors that modulate clot formation or fibrinolytic activity, we measured the levels of fibrinogen and PAI-1. Although the levels of fibrinogen were similar in plasma from septic patients and controls, the levels of PAI-1 were increased in plasma from septic patients containing low or intermediate amounts of CFDNA and markedly elevated in samples with high levels of CFDNA (Table 2).

### Table 2. Levels of CFDNA, α-Dimer, Fibrinogen, and PAI-1 in Healthy Control and Septic Patient Plasmas (n=10 for Healthy Controls; n=20 for Septic Subgroups)

<table>
<thead>
<tr>
<th>Cohort</th>
<th>CFDNA, µg/mL</th>
<th>α-Dimer, µg/mL</th>
<th>Fibrinogen, µg/mL</th>
<th>PAI-1, µg/mL</th>
<th>α2-AP, % of control</th>
<th>TAFI, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.45±0.233</td>
<td>61.2±12.6</td>
<td>4874±78</td>
<td>4.68±2.13</td>
<td>100</td>
<td>65±2.6</td>
</tr>
<tr>
<td>Low</td>
<td>1.72±0.086</td>
<td>44.9±28.7</td>
<td>4712±136</td>
<td>27.23±3.16*</td>
<td>84±7.2</td>
<td>54±1.8</td>
</tr>
<tr>
<td>Intermediate</td>
<td>8.78±0.37*</td>
<td>49.9±15.3</td>
<td>4939±415</td>
<td>32.32±12.05*</td>
<td>97±7.7</td>
<td>54±3.2</td>
</tr>
<tr>
<td>High</td>
<td>17.6±2.6*</td>
<td>20.4±4.7*</td>
<td>5000±63</td>
<td>76.86±3.24*</td>
<td>75±6.5</td>
<td>52±3.8</td>
</tr>
</tbody>
</table>

α2-AP indicates α2-antiplasmin; CFDNA, cell-free DNA; PAI-1, plasminogen activator inhibitor-1; and TAFI, thrombin activatable fibrinolysis inhibitor.

*P<0.05 compared with healthy control plasma.

**Addition of CFDNA to Normal Plasma Delays Fibrinolysis**

To determine the effects of DNA on clot turbidity and lysis, we performed clot lysis assays on control plasma (pooled from 10 healthy volunteers) supplemented with increasing concentrations of exogenous genomic DNA (isolated from buffy coats of healthy volunteers). Addition of 30 or 40 µg/mL CFDNA to control plasma increased maximum absorbance by ≈2-fold (P=0.017 and P=0.007, respectively; Figure 2A), consistent with formation of thicker fibers and produced a 5-fold prolongation of the clot lysis time (P<0.001; Figure 2B). These results are in agreement with our scanning electron microscopy data, demonstrating that the presence of DNA increases...
clot turbidity. With CFDNA addition, clot structure transitioned from a loose collection of fibrin fibers to a densely packed clot structure (Figure 2C–2F). These results suggest that DNA is responsible for the changes in clot structure and lysis observed in plasma from septic plasmas.

We next sought to investigate the effects of DNA size on fibrinolysis. CFDNA fragments of varying sizes were generated by sonication. The size of CFDNA is known to be dependent on the cellular process by which it was liberated; apoptotic cells release a ladder pattern of DNA at $\approx 150$-bp interval, whereas necrotic and NETting neutrophils release high molecular weight fragments >10,000 bp. Whereas CFDNA fragments composed of 300, 1500, or 10,000 bp significantly increased maximum absorbance and prolonged clot lysis times, CFDNA fragments of 150 bp did not (Figure 2A and 2B). This suggests that only DNA fragments >150 bp impair clot lysis.

**CFDNA Alters Clot Structure by Modulating Thrombin Generation**

Thrombin concentrations are known to affect fibrin assembly and clot morphology. High concentrations of thrombin promote the formation of fibrin clots consisting of thin, densely packed fibrin strands. Previously, we demonstrated that the elevated levels of CFDNA in plasma from septic patients promoted thrombin generation. To determine the contribution of DNA-dependent thrombin generation to the observed changes in fibrinolytic activity and clot morphology, thrombin and tPA were added to control plasma samples and samples from septic patients, and maximum absorbance values and lysis times were measured. The addition of exogenous thrombin normalized the differences in maximum absorbance values observed between subgroups (Figure 3A), but clot lysis remained incomplete in samples with intermediate and high levels of CFDNA (Figure 3B). These results were confirmed by clot lysis assays in plasmas, where coagulation was triggered by the addition of batroxobin, a thrombin-like snake venom that only releases FPA (fibrinopeptide A; data not shown). This evidence suggests that promotion of thrombin generation may contribute to the more tightly packed clot morphology, but thrombin levels alone do not seem to be the sole determinant of the impaired fibrinolysis.

**CFDNA Delays Plasmin-Mediated Lysis of Fibrin Clots**

To delineate the mechanism by which elevated concentrations of CFDNA impair fibrinolytic activity, studies were performed

### Table 3. Parameters of Clot Morphology in Septic Patient Plasmas Observed by Scanning Electron Microscopy

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Fiber Diameter, $\mu$m</th>
<th>Density, % pores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.132±0.004</td>
<td>67.41±1.78</td>
</tr>
<tr>
<td>Low</td>
<td>0.190±0.006*</td>
<td>46.39±2.14</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.235±0.005*</td>
<td>35.04±1.21*</td>
</tr>
<tr>
<td>High</td>
<td>0.277±0.008*</td>
<td>64.93±1.80</td>
</tr>
</tbody>
</table>

Results reflect the mean±SE of at least 3 independent determinations. *P<0.05 compared with healthy control plasma.

**Figure 2.** Cell-free DNA (CFDNA) impairs fibrinolysis in normal plasma. Clot lysis assays were performed in normal plasma supplemented with increasing concentrations of CFDNA in the presence of 1 nmol/L tissue-type plasminogen activator (A). Changes in clot structure were measured by observing clot turbidity at $A_{450}$ (B). Changes in clot morphology were subsequently confirmed by scanning electron microscopy in normal control plasma (C), and control plasma supplemented with 5 $\mu$g/mL (D), 15 $\mu$g/mL (E), and 40 $\mu$g/mL (F) of exogenous genomic DNA. *P<0.05 and **P<0.001.
in a purified system. After clotting 5 μmol/L fibrinogen with 5 nmol/L thrombin in the absence or presence of increasing concentrations of CFDNA, fibrinolysis was initiated by the addition of 1 μmol/L Glu-plasminogen and 1 nmol/L tPA. CFDNA significantly delayed tPA-mediated clot lysis times by 2.2-fold when CFDNA concentrations exceeded 20 µg/mL (P=0.002; Figure 4A). Despite this delay, however, CFDNA seemed to have no impact on tPA-mediated conversion of plasminogen to plasmin (Figure 4B).

To explore the possibility that CFDNA impairs the catalytic activity of plasmin directly, experiments were repeated using 1 nmol/L plasmin or trypsin in place of tPA/plasminogen. Whereas CFDNA had no effect on lysis times with trypsin (data not shown) at concentrations of 20 µg/mL, CFDNA prolonged plasmin-mediated lysis times by 1.7-fold (P<0.001; Figure 4C). To confirm that CFDNA impaired clot degradation by plasmin, clots were incubated with plasmin in the absence or presence of 40 µg/mL CFDNA, and at intervals, reactions were stopped and clots were solubilized by the addition of acetic acid and subjected to SDS-PAGE analysis (Figure 4D). Compared with plasmin-mediated fibrin lysis in the absence of CFDNA, the addition of CFDNA reduced fibrin α-chain degradation ≥3-fold after 60 minutes of lysis, as determined by densitometry (P<0.001). Together, this evidence suggests that CFDNA does not affect the ability of tPA to generate plasmin, but rather inhibits fibrinolytic activity by attenuating plasmin activity.

**Interaction of DNA With Fibrinogen, Fibrin, Plasminogen, and Plasmin**

Surface plasmon resonance was used to quantify the interaction of DNA with fibrinogen (Figure 5A) and fibrin (Figure 5B). As shown in the sensorgrams, genomic DNA exhibits rapid association and slow dissociation with both fibrinogen and fibrin. Kinetic analysis of the on- and off-rates demonstrates that genomic CFDNA binds fibrinogen and fibrin with similar affinities (K_d values of 3.2±0.2 and 2.1±0.2 nmol/L, respectively). In contrast, binding of smaller CFDNA fragments (=150 bp in size) to fibrinogen was undetectable (data not shown).

To investigate whether genomic CFDNA is able to compete with fibrinogen/fibrin for plasmin binding, the interaction of Glu-plasminogen or plasmin with immobilized biotinylated-DNA was investigated using surface plasmon resonance. Kinetic analyses of the sensorgrams show that Glu-plasminogen binds DNA with a K_d value of 4.2±0.3 nmol/L (Figure 5C), whereas plasmin binds DNA with a K_d value of 3.5±0.3 nmol/L (Figure 5D). Therefore, DNA binds fibrinogen and plasminogen and may interfere with plasmin-mediated degradation of fibrin clots.

**CFDNA Inhibits Fibrinolysis via the Formation of DNA/Plasmin/Fibrin Ternary Complex**

Although DNA binds both fibrin and plasmin, it was unclear whether CFDNA impairs plasmin-mediated fibrinolysis by competing with plasmin for fibrin binding or whether DNA is able to bind both simultaneously. To examine these possibilities, we performed a fluorometric microplate assay to quantify plasmin binding to clots formed in the presence or absence of CFDNA.

The addition of ≥10 kbp CFDNA fragments to fibrin clots resulted in an increase in f-PPACK-plasmin binding, as indicated by a decrease in fluorescent intensity of the supernatant (Figure 6). Subsequent increases in DNA concentration did not have any additional effect on fluorescent intensity. However, in the presence of DNA fragments of ≥150 bp in size, there was little effect on plasmin binding. Collectively, these data suggest that larger CFDNA fragments are able to bind both plasmin and fibrin simultaneously, thereby forming a ternary complex that hinders the enzymatic activity of plasmin.

**Discussion**

The levels of CFDNA have been reported to be increased in various coagulopathy-associated disease states, including trauma, cancer, stroke, and sepsis. Of particular interest is the ability of CFDNA to potentiate disease pathology through activation of coagulation via the contact pathway. In a previous study, we demonstrated that increased concentrations of endogenous CFDNA correlated with increased endogenous thrombin potential in plasmas obtained from patients with severe sepsis. Yet, in addition to coagulation activation and the downregulation of endogenous anticoagulant
pathways, impairment of the fibrinolytic system plays a central role in the pathogenesis of microvascular thrombosis and organ dysfunction in sepsis.\(^8,9,12,13\) To date, the mechanisms that modulate fibrinolysis in sepsis remain incompletely understood.

This study has revealed 3 major findings. First, we demonstrated that elevations in endogenous CFDNA levels in the plasma of patients with severe sepsis correlate with the production of clots that are resistant to lysis. Only when plasma from septic patients was treated with DNase was fibrinolytic activity restored, thus highlighting the involvement of CFDNA in fibrinolysis. Second, we showed that increased CFDNA concentrations in plasma result in altered clot morphology and that this effect is likely because of potentiation of thrombin generation by CFDNA. Finally, we demonstrated that CFDNA is able to bind both fibrin and plasmin to form a nonproductive ternary complex that results in delayed clot lysis in both plasma and purified systems.

Impaired fibrinolysis in sepsis is well documented and has previously been attributed to the increased levels of PAI-1 because of its release from vascular endothelial cells.\(^8,10,14–16\) Elevations in PAI-1 levels have been shown to correlate with disease severity and poor outcome in patients with sepsis.\(^38–40\)

Previous studies in which lipopolysaccharide was administered to human\(^41,42\) or nonhuman\(^43\) primates demonstrated that endotoxemia produces a temporary increase in tPA activity, and activation of fibrinolysis that is followed by a robust increase in PAI-1 levels that results in an antifibrinolytic state. Interestingly, activation of plasminogen by tPA and subsequent fibrinolytic activity in sepsis patients has been shown to remain ongoing despite increases in PAI-1 as determined by plasma levels of plasmin–α\(_2\)-antiplasmin complexes and D-dimer.\(^39,44,45\) Thus, although the inhibitory effects of PAI-1 may contribute to impaired fibrinolysis in sepsis, there are likely other critical antifibrinolytic mechanisms involved. In this study, although the levels of PAI-1 were determined to be elevated in septic plasmas, inhibition of PAI-1 activity alone was not sufficient to restore fibrinolysis. This evidence suggests that CFDNA and PAI-1 may both contribute to the suppression of fibrinolytic activity observed in septic patients.

Figure 4. Effects of cell-free DNA (CFDNA) on clot formation and lysis in a purified system. A, Fibrin clots were formed with 10 \(\mu\)mol/L fibrinogen (Fg) and 5 nmol/L thrombin in the presence of 1 nmol/L tissue-type plasminogen activator (tPA) and 1 \(\mu\)mol/L Glu-plasminogen. Clot lysis times were assessed by observing turbidity at \(A_{405}\). Lysis time was taken as the time required for absorbance to reach the midpoint between clot formation and clot lysis. B, Plasminogen activation by 1 nmol/L tPA and 1 \(\mu\)mol/L Glu-plasminogen in the presence of increasing concentrations of CFDNA as determined by plasmin (Pn)-specific chromogenic substrate (S-2251) cleavage. C, Clot lysis times of clots where fibrin degradation is mediated by 6 \(\mu\)mol/L Pn. Fibrin clots were formed with 10 \(\mu\)mol/L Fg and 5 nmol/L thrombin. D, Analysis of Pn-mediated fibrin degradation by SDS-PAGE. Identical clots were formed with or without the addition of 50 \(\mu\)g/mL CFDNA. Clot breakdown was halted at various time points by the addition of acetic acid. * \(P<0.05\).
In addition to a delay in clot lysis, we also observed significant alterations in clot structure when CFDNA levels were elevated. There are many factors that contribute to modified clot structure, including pH, ionic strength,\textsuperscript{46} and concentrations of calcium,\textsuperscript{47} fibrinogen,\textsuperscript{48} or dextran.\textsuperscript{49} One of the most important physiological regulators of clot structure is the concentration of thrombin at the time of clot formation.\textsuperscript{35} Subnanomolar concentrations of thrombin are capable of cleaving fibrinopeptides and catalyzing fibrin polymerization. Low thrombin concentrations produce clots that are composed of loosely packed fibrin fibers, whereas high concentrations of thrombin produce tightly packed fibrin clots that are more resistant to lysis.\textsuperscript{35} In our studies, increased CFDNA in plasmas from septic patients correlated with denser fibrin networks. We were able to normalize clot structure by supplementing the plasmas with exogenous thrombin, but this had no effect on clot lysis. Therefore, although CFDNA seems to modulate clot structure by enhancing thrombin generation, changes in clot morphology do not seem to contribute to fibrinolytic resistance.

Recent work by Varju et al.\textsuperscript{50} reported on the effect of histones on clot structure. The addition of at least 250 μg/mL of histone protein to a forming clot resulted in more opaque clots that correlated with thicker fibrin fibers. Histones are often released into the circulation along with CFDNA, particularly during NETosis.\textsuperscript{51} We previously quantified the levels of CFDNA in plasmas from septic patients and found a significant correlation with histone levels. This suggests that CFDNA and histones may work together to modulate clot structure.

Figure 5. Binding of fibrinogen (Fg), fibrin (Fn), plasminogen, and plasmin to DNA as determined by surface plasmon resonance. A, Fibrinogen was covalently linked to carboxyl groups on a CM4 sensor chip. An unmodified carboxymethyl dextran–containing cell served as the reference control. Genomic cell-free DNA (CFDNA) was injected into the flow cells for 400 s to assess binding, followed by buffer injection to assess dissociation. B, Biotinylated Fg was subsequently converted to Fn after multiple injections of 500 nmol/L thrombin. Genomic CFDNA was injected to assess binding to Fn. C, Biotinylated DNA was adsorbed on flow cells containing streptavidin, and 0 to 16 μmol/L of Glu-plasminogen was passed through the flow cells to assess binding. After regeneration, injections were repeated using 0 to 16 μmol/L of Fn (D). Data represent the mean±SD of 3 determinations.

Figure 6. Effect of cell-free DNA (CFDNA) on the displacement of plasmin from fibrin. Fluorescently labeled, active site–inhibited plasmin was incubated with fibrinogen and increasing concentrations of genomic DNA of various lengths. F-PPACK-Pn (fluorescein-D-Phe-Pro-Arg-CMK–inhibited plasmin) displacement was determined by quantifying fluorescent intensity of clot supernatant. *P<0.05, **P<0.01 relative to clots formed with no DNA present.
of circulating DNA-histone complexes in our septic patient plasmas; although the levels were higher than those in healthy controls, they did not exceed 10 µg/mL. Therefore, the low levels of histones in our septic patient plasma samples are unlikely to directly influence clot morphology.

Previous studies suggested that DNA may potentiate plasminogen activation by tPA and urokinase plasminogen activator via a template mechanism that is dependent on ionic strength. As a result, it was suggested that DNA may compete with fibrin for binding plasminogen and tPA to ultimately promote fibrinolysis. The results from our present study support the findings that DNA is able to bind both plasminogen and plasmin with physiologically relevant affinities; however, the cumulative effect of this interaction in plasma correlates with a decrease in clot dissolution. Similarly, Varjua et al recently demonstrated that DNA has a modest effect on tPA-mediated activation of plasminogen, despite a concurrent impairment in fibrinolytic activity. Our studies demonstrate that only large CFDNA fragments are able to bind plasmin and fibrin simultaneously, inhibiting clot lysis through the formation of a nonproductive ternary complex. This observation is supported by recent work by Longstaff et al, who showed that the addition of exogenous DNA extended lysis times in a purified system. The authors concluded that DNA serves to bind large fibrin degradation products and stabilizes the dismantling clot, ultimately prolonging fibrinolysis. Here, we demonstrated that CFDNA binds with high affinity to fibrinogen and fibrin. Therefore, it is likely that larger CFDNA molecules are able to associate with a forming clot and confer additional stability. However, as suggested by visualization of fibrin degradation products in our system (Figure 4), the presence of CFDNA does not simply serve as an adhesive molecule for fibrin degradation products, but rather prevents fibrin from being degraded by plasmin. Thus, the ability of CFDNA to impair plasmin-mediated clot lysis coupled with its potential to stabilize the degrading fibrin scaffold may work in concert to delay fibrinolysis.

Particular emphasis has been placed on the harmful effects of elevated levels of CFDNA in various disease states. Yet, it is important to consider that coagulation activation and subsequent deposition of fibrin at sites of infection represent a critical mechanism of host defense against invading pathogens. In fact, several species of bacterial pathogens have evolved ways to circumvent entrapment by exploiting host plasmin-mediated proteolysis to facilitate their own dissemination. For example, Yersinia pestis directly activates plasminogen via its surface Pla protease, whereas several other species of bacteria potentiate plasmin-mediated fibrinolysis by recruiting plasminogen to their surface, where it is subsequently activated by host tPA.

Similarly, the release of CFDNA in the form of NETs also represents an evolutionarily conserved mechanism of host defense that is essential for infection control. Using a cecal ligation and puncture model of polymicrobial sepsis, Meng et al showed that the dismantling of NETs in vivo by DNase in the early stage of sepsis progression is deleterious, resulting in increased bacterial dissemination and enhanced inflammation. In contrast, we have shown that delayed administration of DNase in mice subjected to cecal ligation and puncture is beneficial, resulting in a reduction of CFDNA-mediated coagulation and a reduction in bacterial dissemination presumably because the bulk of the microbes are already disarmed by NETs. The findings of this study suggest that the CFDNA component of NETs not only traps circulating pathogens, but may also work in tandem with fibrin to prevent systemic dissemination of microbes by limiting plasmin-mediated fibrin degradation. Thus, understanding the balance between the beneficial and deleterious effects of a hypofibrinolytic state in sepsis is critical to the development of therapeutic strategies.

In summary, our studies are the first to examine the effect of CFDNA on fibrinolysis in the context of sepsis. Our findings suggest that CFDNA inhibits fibrinolysis in septic patients by impairing plasmin-mediated fibrin degradation, thereby identifying CFDNA as a potential therapeutic target for sepsis.

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T.J. Gould, T.T. Vu, and A. Stafford performed the experiments. T.J. Gould and Dr Liaw wrote the article. T.T. Vu, D.J. Dwivedi, P. Kim, J.I. Weitz, and Dr Liaw edited the article. T.J. Gould, T.T. Vu, P. Kim, J.I. Weitz, and Dr Liaw designed the research study. We are extremely grateful to Dr Deborah Cook, Ellen McDonald, Nicole Zytaruk, and Bronwyn Cash-Barlow for the recruitment of patients with sepsis in Hamilton, Ontario, Canada.

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Disclosures

None.

References

Extracellular histones are major mediators of death in sepsis.

FB, Esmon NL, Lupu F, Esmon CT. Extracellular histones are major mediators of death through TLR2 and TLR4 in mouse fatal liver injury.

Extracellular DNA traps promote thrombosis.


DeLa Cadena RA, Majfuz-Cruz A, Stadnicki A, Agosti JM, Colman RW, Suffredini AF. Activation of the contact and fibrinolytic systems after intravenous administration of endotoxin to normal human volunteers: correlation with the cytokine profile. Immunopharmacology. 1996;33:231–237.


Varju I, Longstaff C, Szabo L, Farkas ÁZ, Varga-Szabó VJ, Tanká-Salamon A, Machovich R, Kolev K. DNA, histones and neutrophil


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**Significance**

These studies examine the interplay between cell-free DNA (CFDNA) and sepsis pathophysiology by (1) investigating the relationship between CFDNA levels and fibrinolytic activity in sepsis, and (2) determining the mechanisms by which CFDNA modulates fibrinolysis. We demonstrate that elevated levels of CFDNA correlate with impaired fibrinolytic activity in the plasma of severe sepsis patients and that this effect can be reversed on the addition of DNase. The changes in fibrinolytic activity in septic plasmas correlated with changes in clot morphology, a phenomenon that seems to be mediated by increases in thrombin generated by CFDNA itself. In a purified system, CFDNA delays fibrinolysis and does not affect tissue-type plasminogen activator–mediated plasmin generation. Our studies also show that CFDNA is able to bind both plasmin and fibrin to form a nonproductive ternary complex that inhibits plasmin-mediated fibrinolysis. These findings support the concept that CFDNA may be an important therapeutic target in sepsis treatment.
Cell-Free DNA Modulates Clot Structure and Impairs Fibrinolysis in Sepsis
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Materials and Methods

Materials
Human alpha-thrombin (IIa), unfractionated, FXIII-free fibrinogen (Fg), and Glu-Plasminogen (Glu-Pg) were from Enzyme Research Laboratories (South Bend, IN, USA). Plasmin (Pn) and fluorescein-labeled FPR-chloromethylketone (PPACK) were from Haematologic Technologies Inc. (Vermont, USA). Recombinant human DNase I (Pulmozyme® dornase alpha) and alteplase (recombinant single-chain tPA) were from Genentech (San Francisco, CA, USA). RNase was from Promega (Madison, WI, USA). The PAI-1 inhibitory antibody MA-55F4C12 was purchased from Hycult Biotech (Plymouth, PA, USA). Sequencing grade trypsin was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Heparin was purchased from Leo Pharma (Thornhill, ON). H-D-Val-Leu-Lys-p-nitroaniline-dihydrochloride, the plasmin-directed chromogenic substrate S-2251 was from Chromogenix (Bedford, MA, USA). Batroxobin and streptavadin were purchased from Sigma Aldrich (St. Louis, MO, USA). PHOTOPROBE kit (long arm) biotin for nucleic acid labeling was purchased from Vector Laboratories (Burlingame, CA, USA). HOOK-sulfo-NHS-LC-biotin was purchased from G-Biosciences (St. Louis, MO, USA). Plasma levels of D-dimer, PAI-1, and fibrinogen were quantified by immunoassay using kits from Ray Biotech, Inc. (Norcross, GA, USA), Abcam (Cambridge, MA, USA), and Affinity Biologicals (Ancaster, ON), respectively.

Human Sample Collection

Patients and plasma samples
Frozen plasma samples were obtained from an available biobank that contains samples from 400 patients with severe sepsis (DYNAMICS Study, ClinicalTrials.gov Identifier: NCT01355042). The patients were recruited between September 2010 and January 2013 from tertiary care ICUs from 9 centers across Canada. Patients with severe sepsis were identified using the inclusion and exclusion criteria previously described by Dwivedi et al. [10]. The study was approved by the Research Ethics Boards of all participating centers.

Patient blood samples, which were collected within 24 hours of meeting the inclusion criteria for severe sepsis, were processed within 2 hours. Briefly, 9mL of arterial blood was withdrawn from an indwelling catheter and transferred into 15mL polypropylene tube containing 0.5mL of 0.105M buffered trisodium citrate (pH 5.4). After centrifugation at 1500 x g for 10 min at 20°C, platelet poor plasma was harvested and stored in 200µL aliquots at -80°C until used.

Plasma samples from healthy controls
Plasma samples were obtained via venipuncture from 10 healthy adult volunteers who were not receiving any medication at the time of blood collection. There was no attempt
to match controls with cases. The blood was processed as described above, and plasma was pooled and stored in aliquots at -80°C until used.

**DNA isolation and quantification**

To isolate DNA from plasma or buffy coat, samples were subjected to centrifugation at 1500 x g for 10 min to sediment cellular debris and DNA in the supernatant was then isolated using the QIAMP DNA blood mini kit (QIAGEN, Mississauga, ON) according to the manufacturer’s instructions. CFDNA was isolated from 200μL plasma or supernatant and eluted into 200μL elution buffer (10mM Tris-Cl, 0.5mM EDTA, pH 9.0). Concentrations of isolated DNA were determined by measuring absorbance at 260 nm using a BioPhotometer Plus spectrophotometer (Eppendorf, Mississauga, ON) and purity was confirmed by calculating the ratio of absorbance determined at 260 and 280 nm. Integrity of isolated DNA was confirmed by gel electrophoresis on 2% agarose gels. To generate DNA fragments, genomic DNA was subjected to sonication using an M220 Focused-ultrasonicator™ (Covaris, Woburn, MA) according to instrument instructions.

**Plasma Clot Lysis Assays**

Clot formation and lysis times were determined in plasma from septic patients or healthy controls in the absence or presence of 20μg mL⁻¹ DNase/RNase or 20nM of inhibitory PAI-1 antibody MA-55F, which were pre-incubated at 37°C for 4 or 1 h, respectively. Where indicated, ‘ctrl’ refers to control plasma obtained from pooling platelet-poor plasma from 10 healthy volunteers. After adding 50μL of plasma to wells of a flat-bottomed 96-well plate maintained at 37°C, 50μL of a solution containing 1nM tPA and 30mM CaCl₂ in 20mM Tris, pH 7.0 was added. Absorbance was monitored at 405nm for up to 5 h in a SpectraMax M5e plate reader (Molecular Devices, Sunnyvale CA, USA) and clot formation and lysis times were determined as the time to half maximal increase and decrease in absorbance, respectively, as calculated using the instrument software. Where indicated, complete clot lysis was defined as a given sample reaching the half maximal decrease in absorbance during the 5-hour period of observation.

**Clot Lysis Assays in Buffer Systems**

The effect of CFDNA on clot formation and lysis was also examined in a purified system. Aliquots of 10nM thrombin and 1nM tPA were placed separately in wells of a 96-well plate prior to addition of a solution containing 5μM Fg, 1μM Glu-Pg, and 2mM CaCl₂ in 20mM Tris-pH 7.0 in the absence or presence of CFDNA up to 50μg mL⁻¹. In some experiments, 1nM Pn or 5µM trypsin was added in place of tPA and Glu-Pg. In all cases, absorbance was monitored at 405nm for up to 2 h and the clotting and lysis times were determined as described above.
**Evaluation of Plasma Clot Structure using Scanning Electron Microscopy**

After adding 15mM CaCl₂ to 50µL aliquots of plasma from either healthy or septic individuals, 30µL aliquots were removed, deposited on etched glass slides, and incubated for 2 hours at 37°C. Clots were then gently washed 5 times with PBS and submerged and fixed overnight at 4°C in 2% glutaraldehyde. Clots were imaged digitally in 3 different areas using a Tescan Vega II scanning electron microscope (Tescan, USA, PA) at a magnification of 20 000X. Fibrin fiber diameter was determined using ImageJ software (v.1.6). Individual fibers from 10 random areas were measured per image. Fibrin clot porosity was quantified by calculating the number of black pixels relative to total pixels in a given image using Adobe Photoshop CS5 software. For these determinations, a ‘black pixel’ was defined as 75% pixel grayscale pixel intensity, with 0% being equal to white and 100% being equal to completely black.

**SDS-PAGE Analysis of Fibrin Degradation**

Clots were formed and fibrinolysis initiated by incubating 5µM fibrinogen with 5nM thrombin and 1nM plasmin in HEPES-buffered saline (HBS), pH 7.0, containing 0.005% Tween-20 in the absence or presence of DNA up to 50µg mL⁻¹. At various intervals, reactions were stopped and clots were solubilized by the addition 0.1M acetic acid. Aliquots containing 5µg of protein were added to the sample preparation buffer [final concentrations of 1% DodSO₄, 0.05M Tris–HCl (pH 8.0), 0.025M EDTA (pH 8.0), 0.05mg mL⁻¹ 1 bromophenol blue, 10% β-mercaptoethanol and 5% glycerol, 20µL total], and degradation products were resolved by SDS–PAGE using 4–15% gradient gels (Bio-Rad, Mississauga, ON, USA). Gels were stained with Bio-safe Coomassie G-250 stain (Bio-Rad), digitally scanned, and band density was quantified using Image Lab™ Software v.4.1 (Bio-Rad). Band identity was determined by apparent molecular weight[22].

**Plasminogen Activation Assay**

The effect of CFDNA on plasminogen activation by tPA was assessed by incubating 1µM Glu-Pg with 400nM S-2251 at 37°C in wells of a 96-well flat-bottom plate in the absence or presence of CFDNA up to 50µg mL⁻¹. After adding 50nM tPA, S-2251 hydrolysis was monitored at 405nm for 1h. Turbidity was corrected for substrate hydrolysis by subtracting the absorbance measured at 450nm. Corrected absorbance values were then plotted against time-squared and plasminogen activation rates were determined from the linear portions of the plots using the specific activity of plasmin for S-2251, which was determined in a separate experiment to be 1.64 mOD/min/nM. Values in the presence of varying concentrations of CFDNA were then normalized relative to that measured in its absence.
Surface Plasmon Resonance

Binding interactions were studied by surface plasmon resonance (SPR) using a Biacore T200 (GE Healthcare, Piscataway, NJ). FXIII-free Fg in 10mM sodium acetate, pH 5.5, was covalently linked to carboxyl groups on a CM4 sensor chip (GE Healthcare) using an amine coupling kit (GE Healthcare) at a flow rate of 5µL/min until 5000 response units (RU) were attained. Where indicated, immobilized Fg was converted to Fn by injecting 500nM thrombin in HBS containing 10mM CaCl2 and 0.005% Tween-20 at a flow rate of 5µL/min for 60 min. This step was repeated until no further reduction in RU was detected, indicating complete release of fibrinopeptide A (Fp) A and/or FpB. Genomic DNA (isolated from buffy coat of healthy volunteers) was diluted in HBS-Tw and injected at a flow rate of 20 μL/min for 1 min. Flow cells were then washed with HBS-Tw to monitor dissociation. Between runs, flow cells were regenerated with HBS containing 1M NaCl, 10mM EDTA and 0.005% Tween-20.

Alternatively, biotinylated genomic DNA was immobilized to streptavidin-coated flow cells. Flow cells were then washed with 0.02M HEPES, 1M NaCl, 2mM CaCl2, pH 8.0 (HBS) containing 0.005% Tween-20 (Tw). Glu-Pg or Pn diluted in HBS-Tw (pH 8.0) were injected at a flow rate of 20 µL/min for 1 min and flow cells were then washed with HBS-Tw to monitor dissociation. Between runs, flow cells were regenerated with HBS containing 1M NaCl, 10mM EDTA and 0.005% Tween-20, pH 8.0. For each condition, RU values at equilibrium were determined by subtracting the RU values obtained in the control flow cell containing unmodified carboxymethyl-dextran (for Fg/Fn binding studies) or streptavidin (for DNA binding studies). Equilibrium values were then plotted against the starting concentrations of Fg/Fn/DNA and data were fit to a rectangular hyperbola using SigmaPlot v.11.0 software (San Jose, CA, USA) to determine the affinity (Kd) values.

Determination of Pn binding to DNA-containing clots

To study the effects of CFDNA on the Pn-Fn interaction, a fluorometric displacement assay was used. Plasmin was active site labeled by incubation with a 10-fold molar excess of fluorescein-labeled FPR-chloromethylketone (f-PPACK) for 2h at 25°C. After dialysis against phosphate buffered saline (PBS) pH 7.4 four times for 2h at 25°C to remove unincorporated f-PPACK, the material was incubated with S-2251 to confirm complete absence of activity.

The affinity of FITC-labeled Pn for DNA/Fn was determined by measuring unbound Pn in supernatants of clots. Briefly, 3μM Fgn, 9μM f-PPACK-inhibited Pn, and increasing concentrations of genomic DNA (0-500nM) were added to 1.5mL Eppendorf tubes, mixed carefully, and incubated at room temperature for 10min. After adding 10nM thrombin to initiate clotting, clots were incubated for 1 h at 25°C. Fibrin clots were then dislodged from the tubes by vortexing, and collapsed by centrifugation at 16 000 x g.
The supernatant from each tube was collected and 100µL of each sample was added to wells of a 96-well black Costar plate. The fluorescent intensity of the samples was determined using a SpectraMax M5e plate reader (Molecular Devices, Sunnyvale, CA, USA) with excitation and emission wavelengths of 495nm and 520nm, respectively.

Statistical analyses
Values are expressed as mean ± standard error. Significance of differences was determined by one-way ANOVA and Tukey’s pair-wise comparisons or by t-tests using SigmaPlot software. For all analyses, p values less than 0.05 were considered to be statistically significant.