Neutrophil Extracellular Traps Promote Thrombin Generation Through Platelet-Dependent and Platelet-Independent Mechanisms

Travis J. Gould, Trang T. Vu, Laura L. Swystun, Dhruva J. Dwivedi, Safiah H.C. Mai, Jeffrey I. Weitz, Patricia C. Liaw

Objective—Activation of neutrophils by microbial or inflammatory stimuli results in the release of neutrophil extracellular traps (NETs) that are composed of DNA, histones, and antimicrobial proteins. In purified systems, cell-free DNA (CFDNA) activates the intrinsic pathway of coagulation, whereas histones promote thrombin generation through platelet-dependent mechanisms. However, the overall procoagulant effects of CFDNA/histone complexes as part of intact NETs are unknown. In this study, we examined the procoagulant potential of intact NETs released from activated neutrophils. We also determined the relative contribution of CFDNA and histones to thrombin generation in plasmas from patients with sepsis.

Approach and Results—NETs released from phorbol myristate–activated neutrophils enhance thrombin generation in platelet-poor plasma. This effect was DNA dependent (confirmed by DNase treatment) and occurred via the intrinsic pathway of coagulation (confirmed with coagulation factor XI– and coagulation factor XI–depleted plasma). In platelet-rich plasma treated with corn trypsin inhibitor, addition of phorbol myristate–activated neutrophils increased thrombin generation and shortened the lag time in a toll-like receptor-2– and toll-like receptor-4–dependent mechanism. Addition of DNase further augmented thrombin generation, suggesting that dismantling of the NET scaffold increases histone-mediated, platelet-dependent thrombin generation. In platelet-poor plasma samples from patients with sepsis, we found a positive correlation between endogenous CFDNA and thrombin generation, and addition of DNase attenuated thrombin generation.

Conclusions—These studies examine the procoagulant activities of CFDNA and histones in the context of NETs. Our studies also implicate a role for the intrinsic pathway of coagulation in sepsis pathogenesis. (Arterioscler Thromb Vasc Biol. 2014;34:1977-1984.)

Key Words: blood platelets • neutrophils • sepsis

Sepsis is the leading cause of morbidity and mortality in noncoronary intensive care units in the Western world. Severe sepsis, defined as sepsis associated with ≥1 dysfunctional organ, affects ≈750,000 individuals in the United States annually, with an estimated mortality rate of 30% to 50%. Sepsis is often initiated by release of microbial or inflammatory proteins. These structures bind to microorganisms, prevent them from spreading, and ensure a high local concentration of neutrophil granule enzymes to kill bacteria.

CFDNA is the major structural component of NETs, as shown by the ability of DNA-intercalating dyes to stain NETs and by the ability of DNase to dismantle NETs. When activated by microbial or inflammatory stimuli, neutrophils release web-like structures known as neutrophil extracellular traps (NETs), which are composed of CFDNA, histones, and antimicrobial proteins. These structures bind to microorganisms, prevent them from spreading, and ensure a high local concentration of neutrophil granule enzymes to kill bacteria.

CFDNA has emerged as an important link among innate immunity, coagulation, and inflammation. When activated by microbial or inflammatory stimuli, neutrophils release web-like structures known as neutrophil extracellular traps (NETs), which are composed of CFDNA, histones, and antimicrobial proteins. These structures bind to microorganisms, prevent them from spreading, and ensure a high local concentration of neutrophil granule enzymes to kill bacteria.

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.114.304114/-/DC1.

Correspondence to Patricia Liaw, PhD, Thrombosis and Atherosclerosis Research Institute (TaARI), McMaster University, 237 Barton St E, Room C5-107 (5th floor), Hamilton, Ontario L8L 2X2, Canada. E-mail patricia.liaw@tuari.ca

© 2014 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.114.304114
microcirculation results in microvascular thrombosis, leading to tissue hypoxia and endothelial damage.\textsuperscript{11,12} Histones, the other principal component of extracellular traps, are important contributors to the bactericidal and cytotoxic properties of NETs.\textsuperscript{13} Histones are cationic nuclear proteins that associate with DNA to form nucleosomes, the repeating units of chromatin. When injected into mice, histones result in death because of an extreme prothrombotic response, including diffuse microvascular thrombosis, fibrin deposition, platelet aggregation, and thrombocytopenia.\textsuperscript{14} Histone H4 is cytotoxic toward endothelial cells, and blocking histone-mediated cytotoxicity protects mice from endotoxemia.\textsuperscript{14} In purified systems, histones H3 and H4 directly induce platelet aggregation through interactions with toll-like receptors (TLRs) 2 and 4.\textsuperscript{15}

Many of the studies on the procoagulant/proinflammatory properties of CFDNA and histone proteins have examined these components in isolation. However, the majority of CFDNA in plasma is likely histone-bound. Thus, the overall procoagulant effects of CFDNA/histone complexes as part of intact NETs released from activated neutrophils are unknown. Importantly, the interaction between CFDNA and histones may shield many of the pathophysiological effects observed when components are examined in isolation. To address this possibility, we (1) identified the cells responsible for release of CFDNA in blood, (2) compared the capacity of NETs released from activated neutrophils to promote thrombin generation in platelet-poor plasma (PPP) and platelet-rich plasma (PRP), and (3) determined the relative contribution of CFDNA and histones to thrombin generation in plasma from patients with sepsis.

### Materials and Methods

The study design and experimental methods are described in detail in the online-only Supplement.

### Results

**Neutrophils Are the Major Source of Plasma CFDNA Released From Activated Whole Blood**

Previously, we reported that high levels of CFDNA in plasma predicts poor clinical outcome in patients with severe sepsis.\textsuperscript{16} DNA sequence analyses and studies with TLR9 reporter cells suggest that the circulating CFDNA from patients with sepsis is host derived.\textsuperscript{16} In this study, we determined whether neutrophils are the major source of circulating CFDNA released from activated whole blood. Incubation of whole blood with lipopolysaccharide (Figure 1A) or lipoteichoic acid and peptidoglycan (Figure 1B) produced a rapid increase in plasma CFDNA. Similarly, incubation of purified neutrophils with lipopolysaccharide (Figure 1C) or lipoteichoic acid/peptidoglycan (Figure 1D) also resulted in a rapid increase in plasma CFDNA. The amount of CFDNA released by activated neutrophils was similar to that released by activated whole blood, suggesting that neutrophils are the primary source of CFDNA when whole blood is incubated with lipopolysaccharide or lipoteichoic acid+peptidoglycan.

To confirm that the increase in CFDNA from activated whole blood or neutrophils was because of the release of NETs, we performed the studies in the presence of CI-amidine, an inhibitor of NET formation.\textsuperscript{17,18} Specifically, CI-amidine inhibits peptidyl arginine deiminase type IV deimination activity by covalently modifying an active site cysteine on peptidyl arginine deiminase type IV required for chromatin decondensation.

### Figure 1

Cell-free DNA (CFDNA) release from activated neutrophils. Human whole blood was stimulated for varying lengths of time with increasing concentrations of lipopolysaccharide (LPS; A) or lipoteichoic acid (LTA)/peptidoglycan (B), and levels of CFDNA were measured. Isolated human neutrophils were treated with increasing doses of LPS (C) or LTA/peptidoglycan, and levels of CFDNA were measured (n=3 independent experiments). *P<0.05 indicates significance relative to untreated conditions.
As shown in Figure 1, the inclusion of Cl-amidine abrogated the release of CFDNA in whole blood, as well as in purified neutrophils, confirming that the increase in CFDNA is because of the release of NETs. To visualize the released CFDNA, neutrophils mounted on coverslips were imaged after stimulation with increasing concentrations of phorbol-12-myristate-13-acetate (PMA), lipopolysaccharide, and lipoteichoic acid/peptidoglycan. As shown in Figure 2A and Figure II in the online-only Data Supplement, the release of extracellular chromatin was observed in neutrophils stimulated with PMA and bacterial components but not in resting neutrophils.

**NETs Enhance Thrombin Generation Through the Intrinsic Pathway**

To date, there have been no studies that have examined plasma thrombin generation in the presence of intact NETs released from activated neutrophils. To study the effects of NETs on thrombin generation, neutrophils isolated from healthy volunteers were added to PPP and the plasma was then incubated for 30 minutes in the absence or presence of PMA before recalcification and quantification of thrombin generation. In the presence of PMA, the lag time and time to peak thrombin were shorter, and peak thrombin and total thrombin (area under the curve) were higher compared with those measured in the absence of PMA, findings consistent with a procoagulant effect (Figure 2B; Table 1). This activity was diminished with DNase I but not with RNase (data not shown), findings that suggest that the procoagulant activity is mediated by CFDNA.

Although similar results were obtained in coagulation factor VII (FVII)–deficient plasma, when thrombin generation was quantified in FXII- or FXI-deficient plasma, incubation with PMA-activated neutrophils had little effect. The results suggest that the procoagulant activity of CFDNA is mediated via the intrinsic pathway. This concept is supported by the observations that (1) supplementation of FXII- or FXI-deficient plasma with FXII and FXI, respectively, restores procoagulant activity (Figure 2C and 2D), and (2) addition of corn trypsin inhibitor (CTI), a potent and specific inhibitor of FXIIa, to control plasma abolishes procoagulant activity (Figure 2).

**Table 1. Effect of NET Release on Thrombin Generation in Platelet-Poor Plasma**

<table>
<thead>
<tr>
<th></th>
<th>Lag Time, min</th>
<th>Peak IIa, nmol/L</th>
<th>Peak Time, min</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPP only</td>
<td>28±4</td>
<td>58±17</td>
<td>41±5</td>
<td>1188±375</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>24±2</td>
<td>62±6</td>
<td>42±4</td>
<td>1375±140</td>
</tr>
<tr>
<td>NETs</td>
<td>13±1*</td>
<td>225±23*</td>
<td>20±2*</td>
<td>2898±383*</td>
</tr>
</tbody>
</table>

Summary of coagulation parameters for NET release in corn trypsin inhibitor–supplemented PPP. Results reflect the mean±SE of ≥3 determinations. AUC indicates area under the curve; NET, neutrophil extracellular trap; and PPP, platelet-poor plasma.

*P<0.05 compared with PPP.
of PMA-activated neutrophils. In this system, CTI prevents CFDNA-mediated FXII activation and subsequent thrombin generation but is unable to inhibit platelet-mediated (polyphosphate-dependent) FXII activation.19 In the presence of neutrophils, incubation of PRP with PMA shortened the lag time by half and increased peak thrombin compared with the lag time and peak thrombin determined in PPP. The addition of PMA to PRP caused a modest increase in thrombin generation (consistent with the known platelet-activating effects of PMA),20 whereas the addition of PMA-activated neutrophils significantly exacerbated this effect. Compared with PMA-treated PRP, the addition of PMA-activated neutrophils to PRP resulted in a 50% decrease in lag time, accompanied by a significant increase in peak and total thrombin (Figure 3; Table 2). The enhanced procoagulant effect was attenuated with bovine alkaline phosphatase (AP), suggesting that it is mediated by inorganic polyphosphate released from platelets. Thrombin generation in PRP was further enhanced with DNase addition but not with RNase addition. These findings suggest that dismantling of the DNA network of NETs with DNase releases more procoagulant material (presumably histones; Table 2). Addition of TLR2- and/or TLR4-directed inhibitory antibodies attenuated this enhancement (Figure 4A, B), whereas tissue factor–inhibitory antibody HTF-1 or a control IgG had no effect (Table 2). Taken together, these findings suggest that digestion of the DNA network of NETs with DNase exposes the platelet-activating functions of histones.

DNA–Histone Complexes in Plasma From Patients With Severe Sepsis Enhance Thrombin Generation

to determine the physiological relevance of our in vitro studies, we measured plasma levels of DNA–histone complexes in patients with sepsis. Compared with plasma from healthy controls, plasma from patients with severe sepsis contained increased levels of DNA–histone complexes (Figure 5), suggesting that CFDNA circulates in complex with histones. Next, we investigated whether there is a correlation between plasma CFDNA levels and plasma thrombin generation. Plasma samples from severe sepsis patients were divided into those that contained low, intermediate, or high levels of CFDNA as arbitrarily defined as CFDNA levels <5 μg mL⁻¹, 5.0 to 14.9 μg mL⁻¹, and >15 μg mL⁻¹, respectively. Thrombin generation in these samples was then determined in the absence or presence of DNase. In the absence of DNase, there was a direct correlation between CFDNA levels and total thrombin as determined by area under

<table>
<thead>
<tr>
<th>DNase</th>
<th>Lag Time, min</th>
<th>Peak IIa, nmol/L</th>
<th>Peak Time, min</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>PRP</td>
<td>76±2</td>
<td>81±3</td>
<td>63±1</td>
<td>109±11</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>73±1</td>
<td>69±8</td>
<td>85±3</td>
<td>77±16</td>
</tr>
<tr>
<td>PMA</td>
<td>61±16</td>
<td>59±6</td>
<td>101±9</td>
<td>97±12</td>
</tr>
<tr>
<td>NETs</td>
<td>37±2</td>
<td>32±2*</td>
<td>203±13*</td>
<td>234±15*</td>
</tr>
<tr>
<td>NET+Cl-amidine</td>
<td>65±12</td>
<td>72±8</td>
<td>68±9</td>
<td>88±5</td>
</tr>
<tr>
<td>NET+TLR2</td>
<td>41±1</td>
<td>45±9</td>
<td>133±6†</td>
<td>161±7†</td>
</tr>
<tr>
<td>NET+TLR2 and 4</td>
<td>51±2†</td>
<td>56±8†</td>
<td>140±14‡</td>
<td>153±11†</td>
</tr>
<tr>
<td>NET+H3 mAb</td>
<td>44±7</td>
<td>52±3†</td>
<td>177±5</td>
<td>161±3†</td>
</tr>
<tr>
<td>NET+H4 mAb</td>
<td>46±2</td>
<td>55±4†</td>
<td>172±5</td>
<td>132±14†</td>
</tr>
<tr>
<td>NET+H3+H4 mAbs</td>
<td>49±3†</td>
<td>58±12†</td>
<td>143±9†</td>
<td>101±10†</td>
</tr>
<tr>
<td>NET+HTF1</td>
<td>39±2</td>
<td>31±1</td>
<td>210±6</td>
<td>253±13</td>
</tr>
<tr>
<td>NET+IgG</td>
<td>35±2</td>
<td>40±6</td>
<td>202±18</td>
<td>178±8</td>
</tr>
<tr>
<td>NET+AP</td>
<td>89±2†</td>
<td>91±3†</td>
<td>3±1†</td>
<td>8±4†</td>
</tr>
</tbody>
</table>

Summary of coagulation parameters for NET release in corn trypsin inhibitor–supplemented PRP. Results reflect the mean±SE of ≥3 determinations. AP indicates alkaline phosphatase; AUC, area under the curve; mAb, monoclonal antibody; NET, neutrophil extracellular trap; PMA, phorbol-12-myristate-13-acetate; PRP, platelet-rich plasma; and TLR, toll-like receptor. *P<0.05 compared with unstimulated neutrophils.
†P<0.05 compared with NETting neutrophils.
the curve (r=0.6; Figure 6F) and an inverse correlation between CFDNA levels and lag times (r=0.56; Figure 6G). Similarly, higher CFDNA levels were associated with shorter lag times (Figure 6B) and times to peak (Figure 6C), higher peak thrombin values (Figure 6D), as well as greater area under the curve (Figure 6E) relative to control. Incubation with DNase (confirmed by gel electrophoresis; data not shown) attenuated thrombin generation in septic plasma and DNase addition to control plasma reduced thrombin generation to undetectable levels (Figure 6).

The addition of protamine sulfate, a small cationic protein that binds and precipitates DNA, also reduced thrombin generation to undetectable levels (Figure 6A). In addition, although CTI inclusion resulted in an abrogation of thrombin generation, no effect was seen when tissue factor–inhibitory antibody HTF-1 was added to septic plasma and DNase addition to control plasma-reduced thrombin generation to undetectable levels (Figure 6).

Discussion
CFDNAs are extracellular DNA fragments that circulate within peripheral blood. CFDNA circulates at low levels in healthy individuals, with elevated levels observed in an array of clinical conditions including trauma, cancer, stroke, myocardial infarction, and sepsis. In a previous study, we demonstrated that CFDNA seems to have high discriminative power to predict intensive care unit mortality in patients with severe sepsis. Patients with higher plasma concentrations of CFDNA are more likely to face severe complications, such as organ dysfunction/failure and death. As a prognostic indicator of mortality in septic intensive care unit patients, CFDNA alone seems to possess the greatest predictive power, even when combined with existing clinical scoring systems such as MODS (Multiple Organ Dysfunction Score) and APACHE II (Acute Physiology and Chronic Health Evaluation II) scores.

Our present work reveals 4 major findings. First, we identified neutrophil-derived NETs as the most likely source of elevated CFDNA in whole blood exposed to microbial toxins. Second, we demonstrated that intact NETs promote thrombin generation in PPP and that (1) thrombin generation is triggered via the intrinsic pathway, and (2) thrombin generation in PPP is attenuated with DNase I but not RNase. Previous studies have focused on purified NET components or NETs that had been processed before use and thus may lack a physiologically relevant structural integrity. Third, we demonstrated that dismantling the NET scaffold with DNase increases histone-mediated, platelet-dependent thrombin generation. This observation may explain why administration of DNase in septic mice results in organ damage and decreased survival and warrants future studies to explore the therapeutic effects of antihistone therapy in experimental sepsis. Finally, our work demonstrates elevations in indices of thrombin generation in plasma samples from patients with sepsis and implicate an important role of the intrinsic pathway of coagulation in the pathogenesis of sepsis.

For many years, the hemostatic abnormalities in sepsis have been described as an initial hypercoagulable phase driven by aberrant expression of tissue factor, downregulation of endogenous anticoagulant pathways, and impairment of fibrinolysis because of elevations in plasminogen activator inhibitor-1.
Currently, the thrombin generation assay is one of the most extensively used global hemostatic assays used in hemostasis research. However, several groups have reported that patients with sepsis present no signs of systemic hypercoagulability when evaluated with the thrombin generation assay, even in the early stages of sepsis. It should be noted that thrombin generation in previous studies was analyzed in PPP triggered with relipidated tissue factor (ie, via the extrinsic pathway) rather than with CaCl₂ as was done in the present study. Thus, previous studies have overlooked the importance.

Figure 6. Effects of elevated levels of cell-free DNA (CFDNA) in septic plasmas on thrombin generation. Plasmas obtained from patients with severe sepsis were recalcified to initiate coagulation, and thrombin generation was measured as described in Materials and Methods (A). Patients were categorized based on CFDNA concentrations: low CFDNA levels (○) <5 μg/mL; intermediate CFDNA levels (▼) 5.1 to 14.9 μg/mL, and high CFDNA levels (Δ) >15 μg/mL. Deoxyribonuclease (+/−) indicates the presence or absence of a 4-hour deoxyribonuclease (DNase) pretreatment before initiating thrombin generation. Lag time (B), time to peak (C), peak thrombin (D), and area under the curve (AUC; E) analysis was performed with Technothrombin TGA software. Correlation curves for AUC and CFDNA levels (F) as well as lag time and CFDNA levels (G). *P<0.05 and **P<0.01 indicate significance relative to plasma only. Thrombograms shown are representative of 5 independent experiments (n=5 for all subgroups). Ctrl indicates control; ND, no thrombin generation detected; and PS, protamine sulfate.
of the intrinsic pathway of blood coagulation in the hypercoagulable state observed in patients with sepsis.

Given that CFDNA is a potent activator of coagulation, lowering levels of CFDNA may be beneficial to the host in disease states. In humans and mouse models, there is precedence for the therapeutic efficacy of DNase. For example, in patients with cystic fibrosis, a condition often associated with Pseudomonas aeruginosa infection of the lung epithelium, inhalation of recombinant human DNase I reduces the viscosity of purulent sputum and inhibits bacterial biofilm formation. In a mouse model of systemic lupus erythematosus, an autoimmune disease characterized by high circulating DNA levels, intraperitoneal injection of recombinant mouse DNase interferes with the disease process. In the present study, however, we have observed that addition of DNase to NETs actually results in increases in thrombin generation in PRP, suggesting that removal of the CFDNA component of NETs may be detrimental to the host. Consistent with this finding, Meng et al. showed that early digestion of NETs by DNase in a mouse model of sepsis results in advanced sepsis progression accompanied by an increase in mortality. It is possible that, in addition to impairing bactericidal capabilities, early digestion of NETs with DNase exposes histones that are potent activators of platelets and are cytotoxic to vascular endothelial cells. Thus, future studies should aim to better understand the therapeutic potential of degrading NETs in sepsis.

Because histones are presumably exposed when the CFDNA scaffold is degraded by DNase, the absence of thrombin generation in PPP incubated with PMA/DNase-treated neutrophils or purified histones suggests that histones themselves do not trigger the intrinsic pathway of coagulation. It also suggests that neutrophil granular enzymes do not trigger the intrinsic pathway. However, it is possible that neutrophil enzymes promote coagulation by inactivating endogenous anticoagulants. Neutrophil elastase has been shown to degrade antithrombin, and both elastase and cathepsin G (both released during neutrophil degranulation) proteolyze tissue factor pathway inhibitor.

Semeraro et al. have recently demonstrated that purified histones activate platelets throughTLR2 and TLR4, inducing the secretion of inorganic polymer polyP. When thrombin generation was performed in the presence of NETs in CTI-treated PRP (which inhibits CFDNA-mediated but not polyphosphate-mediated contact activation), there was a robust reduction in lag time coupled with an increase in peak thrombin, suggesting that, similar to purified histone proteins, intact NETs are able to activate platelets. This effect was platelet dependent because no thrombin generation was detected when CTI-inhibited PPP was used. However, platelet activation in the presence of NETs was not completely abrogated with TLR2- and TLR4-blocking antibodies, suggesting that alternative mechanisms may also regulate platelet activation in the presence of NETs. Histones have been shown to increase membrane permeability of cells, which contribute to their cytotoxic effects.

In addition to histones, platelet polyP may be another therapeutic target in sepsis. Recent studies have suggested a therapeutic use for AP in the treatment of sepsis-associated organ dysfunction. Two phase II studies demonstrated that parenteral administration of the dephosphorylating enzyme AP to intensive care unit patients with sepsis and associated acute kidney injury improved kidney function and reduced markers of inflammation and kidney injury. The therapeutic efficacy of AP has been attributed to AP-mediated dephosphorylation/detoxification of lipopolysaccharide and dephosphorylation of ATP, a proinflammatory energy molecule released by inflamed renal tissue. In the present study, we have shown that the addition of AP abolished thrombin generation in PRP, suggesting that AP may also exert beneficial effects by impairing platelet polyP-dependent activation of coagulation.

In summary, these studies examine the procoagulant activities of CFDNA and histones in the context of intact NETs. Our studies also implicate a role for CFDNA-mediated activation of the intrinsic pathway of coagulation in the pathogenesis of sepsis. Our findings support the concept that NET components may be important therapeutic targets for the treatment of sepsis.

Acknowledgments
T.J. Gould and T.T. Vu performed the experiments. T. J. Gould and P.C. Liaw wrote the paper. L.L. Swystun, T.T. Vu, D.J. Dwivedi, J.I. Weitz, S.H.C. Mai, and P.C. Liaw edited the paper. J.I. Weitz and P.C. Liaw designed the research study. We are extremely grateful to Dr. Alison Fox-Robichaud, Dr. Deborah Cook, Ellen McDonald, Nicole Zytaruk, and Bronwyn Cashl-Barlow for the recruitment of patients with sepsis in Hamilton, Ontario, Canada.

Sources of Funding
This research was supported in part by a grant-in-aid from the Canadian Institutes for Health Research (grant MOP-106503).

Disclosures
None.

References


Neutrophil Extracellular Traps Promote Thrombin Generation Through Platelet-Dependent and Platelet-Independent Mechanisms

Travis J. Gould, Trang T. Vu, Laura L. Swystun, Dhruva J. Dwivedi, Safiah H.C. Mai, Jeffrey I. Weitz and Patricia C. Liaw

Arterioscler Thromb Vasc Biol. 2014;34:1977-1984; originally published online July 10, 2014; doi: 10.1161/ATVBAHA.114.304114

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/9/1977

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2014/07/10/ATVBAHA.114.304114.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Materials and Methods

Materials
Factor VII-, XI-, FXII-deficient plasmas, human FVII, FXI, and FXII were from Haematologic Technologies (Essex Junction, VT). Corn trypsin inhibitor (CTI) and activated protein C (APC) were from Enzyme Research Laboratories (South Bend, IN). Recombinant human DNase I (Pulmozyme® dornase alpha) was from Genentech (San Francisco, CA). RNase was from Promega (Madison, WI). Heparin was purchased from Leo Pharma (Thornhill, ON). Lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan (PG), phorbol 12-myristate 13-acetate (PMA), bovine alkaline phosphatase (AP), and protamine sulfate (PS) were from Sigma-Aldrich (St Louis, MO). Cl-amidine was purchased from Caymen Chemical (Ann Arbor, MI). Sytox Green and DAPI were purchased from Invitrogen (Burlington, ON). Recombinant human histones H3.1 and H4 were purchased from New England Biolabs (Toronto, ON). Blocking mAbs against human TLR2 (clone T2.5), TLR4 (HTA125), and isotype control (IgG2a) were all purchased from eBiosciences. Inhibitory TF mAb (HTF-1) was purchased from BD Biosciences (Mississauga, ON). Monoclonal inhibitory antibodies against histone H3 (MHIS1947) and histone H4 (MHIS1952) were generously donated by Dr. Charles Esmon (Oklahoma Medical Research Foundation, OK).

DNA isolation and quantification
To isolate DNA from plasma or cell supernatants, 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 200 µL plasma or supernatant and eluted into 200 µL elution buffer (10mM Tris-Cl, 0.5mM EDTA, pH 9.0). The concentrations of isolated DNA was determined by spectrophotometry 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.

Neutrophil isolation
Blood was collected from the antecubital veins of drug- and caffeine-free healthy volunteers into heparin (10 UmL⁻¹). Neutrophils were isolated as previously described[1]. Briefly, whole blood layered onto Lympholyte Poly cell separation media (Cedarlane, Burlington, ON) was subjected to centrifugation for 40 min at 500 x g at 22°C. The neutrophil layer was harvested and residual red blood cells were lysed using RBC lysis buffer (Roche Applied Science, Basel, Switzerland). Neutrophils were then washed and re-suspended in HBSS containing CaCl₂ MgCl₂ (Gibco Invitrogen, Carlsbad, CA) and 2% human serum albumin (Canadian Blood Services, Ottawa, ON). Neutrophil viability of >80% was assessed by flow cytometry using propidium iodide uptake to quantify cell death.

Stimulation of whole blood and neutrophils by bacterial components
Increasing concentrations of LPS (0.1µg/mL or 1µg/mL) or LTA (10µg/mL or
30µg/mL) co-incubated with peptidoglycan (30µg/mL) were added to whole blood or purified neutrophils obtained from healthy volunteers at a concentration of 1 x 10^6 cells/mL. Neutrophil counts were performed from whole blood donors to ensure that neutrophil levels were comparable between the whole blood and purified neutrophil systems. Whole blood or purified neutrophils were subsequently incubated for varying lengths of time at 37°C after which the cells were pelleted, the plasma was collected (by centrifugation at 1500 x g), and CFDNA was quantified.

Visualization of NETs
Isolated neutrophils seeded onto glass coverslips at 1 x 10^6 cells/mL were incubated with 100 nM PMA for 30min at 37°C. PMA was chosen as our NET-stimulator for this and subsequent experiments due to its consistent induction of NET formation and limited capacity to activate platelets (compared to LPS). After washing five times with 1x PBS, total DNA was stained with 5µM DAPI (which passes through intact cell membranes), whereas extracellular DNA was stained with 1µM Sytox Green (which does not cross cell membranes) as previously described[2]. Fluorescent images were acquired using an Olympus BX41 fluorescent microscope at fitted with an Olympus DP72 camera at 20x magnification and analyzed using Slidebook software v.5.0.

Thrombin generation assays
To prepare platelet-poor plasmas (PPP), peripheral venous blood was collected from healthy volunteers into 3.8% trisodium citrate. PPP was prepared by centrifugation at 1500 x g for 10 minutes at room temperature. Simultaneously, platelet-rich plasma (PRP) was prepared by collecting venous blood into citrate supplemented with 50µg/mL CTI. PRP was prepared immediately by centrifugation at 180 x g for 10 minutes at room temperature. Neutrophils were added at a final concentration of 1 x 10^5 cells to 40µL aliquots of PPP or PRP in wells of a 96-well black Costar plate. Where indicated, PPP or PRP was pretreated for 30 minutes with the following enzymes or inhibitors before the addition of neutrophils: DNase I (20µg/mL), RNase (20µg/mL), protamine sulfate (PS; 50µg/mL), anti-TLR2 (200µg/mL), anti-TLR4 (200µg/mL), or anti-H3, anti-H4 (200µg/mL), HTF-1 (10µg/mL), or IgG2a mAbs (50 µg/mL), or bovine AP (10 U/mL). After incubation with 100nM PMA for 30 min at 37°C undisturbed, 15 mM CaCl_2 and 1mM Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland) were added and thrombin generation was monitored using the Technothrombin TGA thrombin generation assay (Technoclone, Vienna, Austria) as previously described[3]. Thrombin generation profiles were analyzed using Technothrombin TGA software (Technoclone).

Quantification of circulating DNA/histone complexes
DNA-histone complex levels were quantified using the Cell Death Detection ELISA Plus kit from Roche Applied Science according to the manufacturer's instructions.
**Gel Electrophoresis**
Extracted DNA was prepared in DNA loading buffer (Thermo Scientific, ON) and 20µL were added to 1% agarose gels for electrophoresis. After staining with ethidium bromide, gels were photographed using UV transillumination.

**Patient plasma samples**
Frozen plasma samples from patients with severe sepsis were collected as part of our ongoing DYNAMICS Study (DNA as a Prognostic Marker in ICU Patients Study; ClinicalTrials.gov Identifier: NCT01355042), an investigator-initiated multi-center prospective observational study that was undertaken to validate CFDNA as a prognostic biomarker in patients with severe sepsis. Patients with severe sepsis, as defined as previously described[4], were recruited between September 2010 and January 2013 from tertiary care ICUs in Hamilton, Ontario. Blood samples were collected within 24 h of enrollment and processed within 2 h. Briefly; 9 mL blood collected from indwelling venous catheters and transferred into 15-mL polypropylene tubes containing 0.5mL of 0.105M buffered trisodium citrate (pH 5.4). After centrifugation at 1500 x g for 10 min at 20°C, plasma was harvested and stored in aliquots at -80°C. The study was approved by the Research Ethics Board of McMaster University and Hamilton Health Sciences, Hamilton,(REB approval 10-532). Signed informed consent was obtained from all patients or substitute decision-makers) or from the healthy controls before blood collection.

**Plasma samples from healthy volunteers**
Using the same methods, plasma samples were obtained from 10 healthy adult volunteers who had been free of medication for at least 48 h. No attempt was made to age or sex match controls with cases.

**Statistical analyses**
Statistical analysis was performed on experiments with an n = 3 or greater. Values are expressed as means ± standard error. Significance of differences was determined by one-way ANOVA and Tukey’s pair-wise comparisons or by t-tests using SIGMAPLOT Software (San Jose, CA, USA).
References


Supplement Material

Supplemental Table I. Indices of thrombin generation in PPP with purified NETs components and their inhibitors.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lag Time (min)</th>
<th>Peak Thrombin (nM)</th>
<th>Peak Height (min)</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>29 ± 3</td>
<td>156 ± 8</td>
<td>33 ± 3</td>
<td>1910 ± 134</td>
</tr>
<tr>
<td>DNA</td>
<td>20 ± 2 *</td>
<td>193 ± 13 *</td>
<td>25 ± 4 *</td>
<td>3007 ± 142 *</td>
</tr>
<tr>
<td>DNAse</td>
<td>58 ± 3 *</td>
<td>23 ± 11 *</td>
<td>62 ± 2 *</td>
<td>814 ± 187</td>
</tr>
<tr>
<td>DNA + DNAse</td>
<td>56 ± 1 *</td>
<td>16 ± 10 *</td>
<td>65 ± 1 *</td>
<td>959 ± 144</td>
</tr>
<tr>
<td>H3</td>
<td>25 ± 4</td>
<td>172 ± 20</td>
<td>27 ± 4</td>
<td>1927 ± 180</td>
</tr>
<tr>
<td>MAbH3</td>
<td>29 ± 2</td>
<td>161 ± 9</td>
<td>33 ± 3</td>
<td>1957 ± 149</td>
</tr>
<tr>
<td>H3 + mAbH3</td>
<td>27 ± 6</td>
<td>152 ± 6</td>
<td>32 ± 4</td>
<td>1845 ± 181</td>
</tr>
<tr>
<td>H4</td>
<td>31 ± 1</td>
<td>147 ± 8</td>
<td>30 ± 4</td>
<td>2056 ± 88</td>
</tr>
<tr>
<td>MAbH4</td>
<td>24 ± 4</td>
<td>162 ± 17</td>
<td>28 ± 5</td>
<td>1836 ± 59</td>
</tr>
<tr>
<td>H4 + mAbH4</td>
<td>30 ± 2</td>
<td>170 ± 3</td>
<td>28 ± 4</td>
<td>1748 ± 30</td>
</tr>
<tr>
<td>APC</td>
<td>28 ± 4</td>
<td>167 ± 11</td>
<td>29 ± 4</td>
<td>1882 ± 31</td>
</tr>
<tr>
<td>H3 + APC</td>
<td>28 ± 2</td>
<td>171 ± 6</td>
<td>27 ± 2</td>
<td>1896 ± 34</td>
</tr>
<tr>
<td>H4 + APC</td>
<td>30 ± 1</td>
<td>156 ± 4</td>
<td>28 ± 1</td>
<td>2020 ± 48</td>
</tr>
</tbody>
</table>

Summary of coagulation parameters for purified NET components in PPP. Results reflect the mean ± SE of at least 3 determinations. Asterisks denote p < 0.05 compared with untreated PPP.

Supplemental Table II. Indices of thrombin generation in PRP with purified NETs components and their inhibitors.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lag Time (min)</th>
<th>Peak Thrombin (nM)</th>
<th>Peak Height (min)</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>62 ± 2</td>
<td>151 ± 10</td>
<td>72 ± 5</td>
<td>2714 ± 289</td>
</tr>
<tr>
<td>DNA</td>
<td>53 ± 10</td>
<td>133 ± 12</td>
<td>64 ± 10</td>
<td>2533 ± 391</td>
</tr>
<tr>
<td>DNAse</td>
<td>63 ± 2</td>
<td>141 ± 11</td>
<td>78 ± 4</td>
<td>2539 ± 324</td>
</tr>
<tr>
<td>DNA + DNAse</td>
<td>53 ± 5</td>
<td>146 ± 6</td>
<td>69 ± 6</td>
<td>2735 ± 77</td>
</tr>
<tr>
<td>H3</td>
<td>8 ± 1 *</td>
<td>264 ± 3 *</td>
<td>23 ± 5 *</td>
<td>4450 ± 193 *</td>
</tr>
<tr>
<td>MAbH3</td>
<td>61 ± 2</td>
<td>152 ± 15</td>
<td>78 ± 7</td>
<td>2641 ± 253</td>
</tr>
<tr>
<td>H3 + mAbH3</td>
<td>56 ± 5</td>
<td>124 ± 15</td>
<td>89 ± 8</td>
<td>2307 ± 276</td>
</tr>
<tr>
<td>H4</td>
<td>7 ± 1 *</td>
<td>280 ± 20 *</td>
<td>22 ± 2 *</td>
<td>4295 ± 234 *</td>
</tr>
<tr>
<td>MAbH4</td>
<td>52 ± 11</td>
<td>144 ± 16</td>
<td>63 ± 12</td>
<td>2678 ± 385</td>
</tr>
<tr>
<td>H4 + mAbH4</td>
<td>62 ± 7</td>
<td>115 ± 17</td>
<td>79 ± 8</td>
<td>1943 ± 264</td>
</tr>
<tr>
<td>APC</td>
<td>60 ± 2</td>
<td>141 ± 11</td>
<td>73 ± 3</td>
<td>2573 ± 147</td>
</tr>
<tr>
<td>H3 + APC</td>
<td>69 ± 5</td>
<td>140 ± 17</td>
<td>78 ± 4</td>
<td>2433 ± 131</td>
</tr>
<tr>
<td>H4 + APC</td>
<td>8 ± 1 *</td>
<td>243 ± 61 *</td>
<td>27 ± 4 *</td>
<td>3970 ± 153 *</td>
</tr>
</tbody>
</table>

Summary of coagulation parameters for purified NET components in PRP. Results reflect the mean ± SE of at least 3 determinations. Asterisks denote p < 0.05 compared with untreated PRP.
Supplemental Figure I. Electrophoretic size comparison of CFDNA from various sources in a 1% agarose gel. CFDNA was purified from 200µL of plasma or cell supernatant using the QIAamp DNA mini Blood Kit (QIAGEN, Valencia, CA). The DNA was resuspended in 200µL of Tris-EDTA buffer and 10µg of DNA was loaded per lane. Lanes 1-3 contain CFDNA isolated from septic patient plasma. Lanes 4-6 contain CFDNA isolated from LPS-treated whole blood. Lanes 7-9 contain CFDNA isolated from LPS-treated peripheral neutrophils.
Supplemental Figure II. Concentration- and time-dependent release of NETs from human neutrophils stimulated with LPS and PMA. Human neutrophils were incubated with increasing concentrations of PMA or LPS for 30, 60, and 120 minutes. NET formation was visualized by staining both intra- and extracellular DNA was with DAPI (blue) and Sytox Green (green), respectively. NETs were observed at 20x magnification using an Olympus DP72 camera at 20x magnification and analyzed using Slidebook software v.5.0.