Cellular Procoagulant Activity Dictates Clot Structure and Stability as a Function of Distance From the Cell Surface

Robert A. Campbell, Katherine A. Overmyer, C. Robert Bagnell, Alisa S. Wolberg

Background—Thrombin concentration modulates fibrin structure and fibrin structure modulates clot stability; however, the impact of localized, cell surface-driven in situ thrombin generation on fibrin structure and stability has not previously been evaluated.

Methods and Results—Human fibroblasts were incubated with factors Xa, Va, prothrombin and fibrinogen, or plasma. Fibrin formation, structure, and lysis were examined using laser scanning confocal microscopy and transmission electron microscopy. In situ thrombin generation on the cell surface produced clots with a significantly denser fiber network in a 10-μm region proximal versus distal to (40 to 50 μm) the cell surface. This morphology was not altered by addition of integrin-blocking RGDS peptide and was not apparent in clots made by exogenous thrombin addition, suggesting that spatial morphology was dictated predominantly by localized thrombin generation on the fibroblast surface. The fibrin network lysed more rapidly distal versus proximal to the cell surface, suggesting that the structural heterogeneity of the clot affected its fibrinolytic stability.

Conclusions—In situ thrombin generation on the cell surface modulates the three-dimensional structure and stability of the clot. Thrombus formation in vivo may reflect the ability of the local cell population to support thrombin generation and, therefore, the three-dimensional structure and stability of the fibrin network. (Arterioscler Thromb Vasc Biol. 2008;28:2247-2254.)

Key Words: coagulation ■ fibrin clot structure ■ thrombin generation ■ fibrinolysis

After vascular injury, thrombin-catalyzes the enzymatic conversion of soluble fibrinogen to an insoluble fibrin network. The fibrin network stabilizes the primary platelet plug, enabling it to withstand the rigors of blood flow during wound healing. A growing number of studies suggest that abnormal fibrin structure makes clots overly stable or friable during this process, contributing to an individual’s risk of thrombosis and embolism.1–4

Mechanisms of fibrin production and clot assembly have been elucidated primarily from studies in which a specific amount of thrombin is added to purified fibrinogen. These studies have shown that the thrombin concentration present at the time of fibrin gelation influences fibrin clot structure.5–9 Low thrombin concentrations produce porous clots composed of thick fibrin fibers, whereas high thrombin concentrations produce clots composed of a dense network of thin fibers. Additional studies have correlated fibrin architecture with the resistance of a clot to mechanical and fibrinolytic disruption.10–11 Clots composed of a dense network of thin fibrin fibers are more resistant to fibrinolysis compared to clots composed of a porous network of thick fibers. In vivo, however, thrombin generation is a dynamic process in which the concentration of thrombin actively changes during the reaction course in accord with the local conditions. Importantly, and in contrast to in vitro experiments initiated by the addition of exogenous thrombin to fibrinogen, thrombin generation in vivo is localized to a cell surface. A recent study using real-time videomicroscopy demonstrated that the procoagulant activity of tissue factor-bearing cells regulates the fibrin formation rate up to 200 μm from the cell surface but does not modulate clot growth further from the cell.12 These findings suggest that the majority of thrombin generated on the initiating cell is spatially restricted to the region surrounding the cell, with limited diffusion to distal regions of the reaction milieu. Thus, different regions of the growing thrombus are exposed to different concentrations of thrombin, as a function of time and distance from the source of prothrombinase activity. Together, these findings suggest that when thrombin generation is localized to a cell surface, the resulting fibrin structure and stability reflects the specific procoagulant activity of the local cells, as well as the spatial location of the fibrin with respect to the cell surface.

In the current study, we examined fibrin structure and stability arising as a function of in situ thrombin generation on a cell surface. To specifically focus on the role of the cell surface in determining clot structure, reactions were per-
formed using purified proteins or plasma in the absence of platelets or microparticles. We observed that fibrin architecture reflected the distance of fibrin from the cell surface, but not RGD-mediated interactions between the cell surface and the fibrin network. This spatial heterogeneity in clot structure caused differential rates of clot formation and lysis proximal versus distal to the cell surface. Our results suggest that in situ thrombin generation on a cell surface modulates clot structure and stability in three dimensions.

**Materials and Methods**

### In Situ Thrombin Generation and Fibrin Clot Formation

The source of proteins and materials used is listed in the Supplemental Data section (available online at http://atvb.ahajournals.org). Fibroblasts were washed with PBS and immediately incubated with factors Xa, Va, and CaCl₂ (1 mmol/L, 5 mmol/L, 5 mmol/L, final, respectively) and prothrombin (0.014 μmol/L or 1.4 μmol/L, final, as indicated) in 20 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.4), 150 mmol/L NaCl (HBS). Thrombin generation was measured by quenching 10 μL aliquots of supernatant with 90 μL of HBS containing 10 mmol/L ethylenediamine tetraacetic acid (EDTA) and 1 mg/mL bovine serum albumin (BSA). Quenched reactions were diluted 1:10 in HBS containing 1 mmol/L EDTA and 0.5 mmol/L Chromozym Th. The substrate cleavage rate (change in absorbance at 405 nm versus time) was measured using a SpectraMax 340PC microplate reader (Molecular Devices). Clot formation was monitored by turbidity. Clots were formed as above in the presence of 0.024 g of AlexaFluor-488-labeled fibrinogen. In reactions in which clot formation was initiated by exogenous thrombin addition, fibrinogen, CaCl₂, and 2 mmol/L thrombin (final) were premixed and immediately added to the cells.

### Structural Analysis by Laser Scanning Confocal Microscopy

Cells were grown to confluence on LabTek II Chamber uncoated #1.5 cover-glasses (Nalge Nunc International). Clots were formed as above with the addition of 10 μg AlexaFluor-488-labeled fibrinogen/150 μL sample. Clot formation was allowed to proceed until a constant final turbidity was reached in separate, parallel reactions. In experiments performed in the absence of cells, chambers were pretreated with SigmaCote (Sigma) to eliminate surface effects of glass on the polymerizing fibrin. For lysis studies, cells were grown to confluence on MatTek uncoated #1.5 glass 35-mm dishes (Ashland). Clots were formed as above in the presence of 0.024 μmol/L plasmin (final). Chambers were immediately scanned at the cell surface and 50 μm above the cell surface and then every 15 seconds at each location until complete lysis. Conditions for laser scanning confocal microscopy (LSCM) and analysis are described in the Supplemental Data section.

### Clot Formation in Normal Pooled Plasma

Cells were grown to confluence on sterile glass chamber slides for LSCM and sterile Thermaxon tissue culture cover slips (Miles Laboratories) or in 8-well Permanox chamber slides (Nalge Nunc International) for transmission electron microscopy (TEM). Cells were washed with PBS and clots were formed by incubating fibroblasts with recalcified (10 mmol/L, final) platelet-free plasma (PPF) plus 10 μg of AlexaFluor-488-labeled fibrinogen for two hours. Conditions for TEM are described in the Supplemental Data section.

### Statistical Methods

Pair-wise comparisons were performed using a Student t test; P<0.05 was considered significant.

### Results

The Thrombin Generation Rate Modulates the Onset and Rate of Fibrin Clot Formation

We incubated human fibroblasts with factors Xa and Va and varied the prothrombin concentration to modulate the thrombin generation rate. We monitored thrombin activity with a chromogenic substrate and observed a 41-fold increase in the thrombin generation rate in the presence of 1.4 versus 0.014 μmol/L prothrombin (P<0.007) (Table 1, supplemental Figure IA). To determine the effect of the thrombin generation rate on clot formation, plasma fibrinogen (2 mg/mL, final) was included in reactions and clot formation was monitored by turbidity. Clot formation during high thrombin generation rates (1.4 μmol/L prothrombin) exhibited a shortened onset and 2.2-fold increased polymerization rate compared to clot formation during low thrombin generation rates (Table 1, supplemental Figure IB). Interestingly, despite the significant difference in onset and fibrin polymerization rate, clots formed during high thrombin generation rates had only slightly lower final turbidities than clots formed during low thrombin generation rates (Table 1, supplemental Figure IB). Such a nonlinear relationship between fibrin polymerization rate and final turbidity has been observed in previous studies performed in the presence and absence of cells. Nonetheless, because the onset and polymerization rate are indicative of fibrin structure, these measurements suggested that clots formed during high thrombin generation rates had a different structure than clots formed during low thrombin generation rates.

### Cells Modulate Fibrin Structural Characteristics in Three Dimensions

We next examined the fibrin structure of clots formed during in situ thrombin generation on the cell surface using LSCM. LSCM permits the investigation of unfixed, fully hydrated samples; data from this technique can be used to recreate three-dimensional images permitting structural analysis as a function of focal depth within the sample. LSCM enabled us to examine interclot differences in structure as a function of prothrombinase activity, as well as intraclot differences in structure as a function of spatial location within the clot.

As anticipated from the polymerization data, clots formed during high thrombin generation rates had ≈15% thinner
fibers and a 14% denser fibrin network than clots formed during low thrombin generation rates (compare Figure 1a and 1d with 1b and 1e, respectively; Table 2). Remarkably, however, the network density was ~25% higher in regions proximal (0 to 10 \( \mu \text{m} \)) versus distal (40 to 50 \( \mu \text{m} \)) to the cell surface, each image is from one experiment, representative of 3 to 6 independent experiments. Darker areas show increased fibrin density. Bar=10 \( \mu \text{m} \).

**Figure 1.** In situ thrombin generation on the cell surface modulates clot structure in three dimensions. Clots were formed as described in the Methods in the absence (a through f) or presence (g through i) of RGDS peptide. Three-dimensional projections show clot architecture in 10- \( \mu \text{m} \) sections at (0 to 10 \( \mu \text{m} \)) and above (40 to 50 \( \mu \text{m} \)) the cell surface. Each image is from one experiment, representative of 3 to 6 independent experiments. Darker areas show increased fibrin density. Bar=10 \( \mu \text{m} \).

**Table 2.** Fibrin Structure in 10- \( \mu \text{m} \) Sections 0–10 \( \mu \text{m} \) and 40–50 \( \mu \text{m} \) From the Cell Surface

<table>
<thead>
<tr>
<th>Condition</th>
<th>Density (0 to 10 ( \mu \text{m} ))</th>
<th>Density (40 to 50 ( \mu \text{m} ))</th>
<th>Fiber Diameter (0 to 10 ( \mu \text{m} ))</th>
<th>Fiber Diameter (40 to 50 ( \mu \text{m} ))</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.014 ( \mu \text{mol/L} )</td>
<td>0.763±0.025</td>
<td>0.568±0.048</td>
<td>0.300±0.024</td>
<td>0.311±0.032</td>
<td>NS</td>
</tr>
<tr>
<td>0.014 ( \mu \text{mol/L} ) + RGDS</td>
<td>0.720±0.027</td>
<td>0.569±0.020</td>
<td>ND</td>
<td>ND</td>
<td>...</td>
</tr>
<tr>
<td>( \bar{P} ) (±RGDS)</td>
<td>NS</td>
<td>NS</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>1.4 ( \mu \text{mol/L} )</td>
<td>0.856±0.008</td>
<td>0.662±0.052</td>
<td>0.263±0.002</td>
<td>0.268±0.007</td>
<td>NS</td>
</tr>
<tr>
<td>1.4 ( \mu \text{mol/L} ) + RGDS</td>
<td>0.866±0.042</td>
<td>0.607±0.054</td>
<td>ND</td>
<td>ND</td>
<td>...</td>
</tr>
<tr>
<td>( \bar{P} ) (±RGDS)</td>
<td>NS</td>
<td>NS</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>2 nmol/L thrombin</td>
<td>0.715±0.037</td>
<td>0.700±0.060</td>
<td>0.313±0.013</td>
<td>0.311±0.014</td>
<td>NS</td>
</tr>
<tr>
<td>2 nmol/L thrombin + RGDS</td>
<td>0.683±0.012</td>
<td>0.663±0.040</td>
<td>ND</td>
<td>ND</td>
<td>...</td>
</tr>
<tr>
<td>( \bar{P} ) (±RGDS)</td>
<td>NS</td>
<td>NS</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

### In Situ Thrombin Generation on Fibroblasts Modulates Fibrin Structural Characteristics Independently of RGD-Binding Integrins

The structural heterogeneity of clots formed over fibroblasts suggested that localization of thrombin generation on the cell surface and/or cellular interactions with the fibrin network influenced the clot’s structure. We therefore examined the influence of cellular receptors on fibrin structure in these clots. Previous studies have shown that cellular integrins bind fibrinogen, but that the \( \alpha \beta 3 \) and \( \alpha \beta 3 \) integrins can directly modulate fibrin structure on human umbilical vein endothelial cells (HUVECs) and platelets, respectively.

Because the fibroblasts used in our study expressed both \( \alpha \beta 3 \) subunits (data not shown), we examined the influence of \( \alpha \beta 3 \) and other RGD-binding integrins on fibrin architecture by comparing clots formed in the presence and absence of the integrin-blocking peptide, RGDS (500 \( \mu \text{mol/L} \), final). This concentration of RGDS peptide inhibited platelet aggregation but did not alter the rates of thrombin generation or fibrin polymerization (data not shown). As seen in the absence of RGDS, the mean network density was ~25% higher in regions proximal (0 to 10 \( \mu \text{m} \)) versus distal (40 to 50 \( \mu \text{m} \)) to the cell surface (Table 2). The mean network density was identical with and without RGDS (compare Figure 1d and 1e with 1g and 1h, respectively; Table 2), suggesting that neither \( \alpha \beta 3 \), \( \alpha \beta 3 \), nor other RGD-binding integrins caused the spatially-dependent morphology seen in clots formed during in situ thrombin generation.

To further confirm that RGD-binding integrins did not influence fibrin structure in clots formed on fibroblasts, we triggered clot formation with the addition of a single, monodispersed thrombin concentration to a fibrinogen/calcium solution. These reactions bypass in situ thrombin generation, so that fibrin structure arises from a uniform thrombin concentration distributed throughout the reaction. Previous studies of clots formed over HUVECs and platelets demonstrated that integrin-mediated influences on fibrin structure are detectable after exogenous thrombin addition. Under these conditions, the mean network density in regions proximal (0 to 10 \( \mu \text{m} \)) and distal (40 to 50 \( \mu \text{m} \)) to the cell surface were indistinguishable (Figure 1c and 1f and Table 2), and addition of RGDS peptide did not alter the fibrin structure.
appearance (compare Figure 1f and 1i; Table 2). These findings suggest that in situ thrombin generation on the cell surface induced the formation of spatially-dependent fibrin morphology independent of RGD-binding integrins.

In Situ Thrombin Generation on Fibroblasts Modulates Fibrin Structure

To precisely determine the distance over which cells influence fibrin structure, we quantified fiber diameter and network density in successive images recorded as the focal plane of the LSCM progressed from 0 to 10 and 40 to 50 μm from the cell surface (Figure 2). As seen in the 10-μm sections, fiber diameter measurements did not change as a function of distance from the cell surface in any clots examined (supplemental Figure II), possibly reflecting LSCM resolution limits. Conversely, clots formed during in situ thrombin generation exhibited a significant decrease in network density (48%±10.7% and 51%±2.9% for 0.014 μmol/L and 1.4 μmol/L prothrombin, respectively) between 0 and 10 μm from the cell surface, and a minor further decrease in network density between 40 and 50 μm from the cell surface (Figure 2). The morphology was seen in both the presence and absence of RGDS peptide. Surprisingly, although the global network was similar proximal and distal to the cell surface in clots formed by exogenous thrombin addition (Figure 1 and Table 2), we did find a local decrease in network density (≈11%) between 0 and ≈4 μm in these clots (Figure 2), in both the presence and absence of RGDS peptide (supplemental Figure III). To confirm that the decrease was not attributable to nonspecific chamber surface effects on the fibrin network, we formed clots in the absence of cells via exogenous thrombin addition to a fibrinogen/calcium solution.

Under these conditions, clots showed no change in network density from 0 to 50 μm from the surface (Figure 2). Together, these findings suggest that fibroblasts modulate fibrin structure over a short distance (<4 μm) via a non-RGD-dependent receptor; however, thrombin generation is the predominant determinant of fibrin structure in these clots.

In Situ Thrombin Generation on a Cell Surface Modulates the Rates of Fibrin Formation and Lysis as a Function of Distance From the Cell Surface

Because the thrombin concentration modulates the fibrin formation rate and resistance to fibrinolysis,5–11 we explicitly determined how in situ thrombin generation on the cell surface affected the spatial and temporal aspects of fibrin formation and lysis. We incubated fibroblasts with purified prothrombinase and included plasmin at the reaction start to uniformly disperse plasmin throughout the clotting milieu. In this assay, fibrin formation competes with lysis, and fibrin formation and lysis occur as a function of the thrombin generation rate and network quality.8,15,26 During low thrombin generation rates (0.014 μmol/L prothrombin), fibrin formed proximal, but not distal to the cell surface within 50 seconds (Figure 3, supplemental Movies IVA and IVB). After 2 minutes, fibrin was present proximal and distal to the cell surface. Fibrin distal to (50 μm above) the cell surface lysed 328±82 seconds before lysis at the cell surface. During high thrombin generation rates (1.4 μmol/L prothrombin), we observed immediate fibrin formation near the cell surface <15 seconds after clotting was initiated (Figure 3 and supplemental Movies IVC and...
As in reactions with low thrombin generation, fibrin appearance proximal to the surface preceded that distal to the cell surface, and fibrin lysed above the cell surface 449 seconds before lysis near the cell surface. Interestingly, the lysis rate (decrease in fiber number/m² versus time) was higher near versus distal to the cell surface, consistent with prior observations that fibers in clots with a tight network lyse more quickly than those in a loose network. In contrast, clots produced by exogenous thrombin addition demonstrated essentially simultaneous fibrin formation through the chamber at 50 seconds (Figure 3). The large variability in clots formed by exogenous thrombin addition likely results from the technical challenge of initiating clot formation by thrombin addition; fibrin formation begins as the solution is being transferred, causing variability in clot structure. Regardless, this control experiment shows that differences in the rates of fibrin formation and lysis proximal versus distal to the cell surface were not simply attributable to differences in the plasmin distribution or release of plasmin inhibitors from the cell. Together these findings demonstrate that thrombin generation on a cell surface increases fibrin formation and resistance to lysis near the cell surface versus distal regions within the clot.

Cells Modulate the Network Density of Plasma Clots

Finally, we examined whether in situ thrombin generation on fibroblasts modulated the three-dimensional structure of plasma clots. Clots were formed as described in Methods, and fibrin density was examined using LSCM and TEM. As depicted in Figure 4A, plasma clots exhibited denser fibrin network near the fibroblast surface versus 50 m above the cell surface. The denser network was not abolished by addition of RGDS peptide, suggesting RGDS-binding integrins did not mediate this interaction (Figures 4A and 4B). TEM confirmed these findings, demonstrating 4-fold more fibers proximal (≈1 μm) versus distal to (≈5 μm away) the cell surface (Figure 4C and 4D). In contrast, clots formed in the absence of cells (via slow contact initiation) demonstrated a uniform distribution of thicker fibers. These findings support our LSCM observations and demonstrate that in situ thrombin generation on a cell surface modulates plasma clot structure.

Discussion

Previous studies have made three basic observations with regard to fibrin clot formation and stability. First, blood coagulation is spatially nonuniform, with extrinsic and intrinsic activities differentially modulating the initiation and propagation of fibrin formation in space. Second, the concentration of thrombin present during fibrin gelation dictates the architecture of the fibrin clot. Third, the fibrin clot quality influences its susceptibility to fibrinolysis. Together these observations suggest that when thrombin generation is localized to a cell surface, the structure and stability of the resulting clot depends on cellular procoagulant...
contrast that previously seen on platelets and HUVECs, these subunits on fibrin structure. This lack of integrin effect but may reflect decreased relative density of integrins on the cell surface is accelerated by lipid, of thrombin generation at and above the cell surface. Thrombogenous morphology is likely attributable to differential rates of fibrin clot formation, structure, and lysis. Our findings demonstrate that in situ thrombin generation on a cell surface causes the formation of a structurally heterogeneous clot, with a denser fibrin network that is resistant to fibrinolysis proximal to the cell surface compared to distal regions of the clot. These novel findings demonstrate the importance of the cell as a site of localized procoagulant activity and the location within the clot with respect to the cell surface. In the current study, we examined the influence of in situ thrombin generation on a cell surface on the spatial dynamics of fibrin clot formation, structure, and lysis. Our findings demonstrate that in situ thrombin generation on a cell surface causes the formation of a structurally heterogeneous clot, with a denser fibrin network that is resistant to fibrinolysis proximal to the cell surface compared to distal regions of the clot. These novel findings demonstrate the importance of the cell as a site of localized procoagulant activity in determining clot structure and stability.

The predominant mechanism causing the spatially-heterogeneous morphology is likely attributable to differential rates of thrombin generation at and above the cell surface. Thrombin generation at the cell surface is accelerated by lipid, which offers a ∼6-fold increase in the Vmax relative to the absence of lipid (at sites distal to the surface). Additionally, both mathematical simulations and in vitro studies predict that the diffusional limit of procoagulant enzymes through the fibrin/platelet layer limits thrombosis height. Our experiments were performed under static conditions (lack of flow), however this morphology may be enhanced by the removal of thrombin distal to the cells by flowing blood. The movement of thrombin away from the cell surface is likely limited directly by the diffusion rate and indirectly by thrombin binding sites on fibrin, further impeding its progress through the gel matrix. Thus, the influence of the fibrin network quality on thrombus growth is likely to be complex. Although the fibroblasts used in our study express fibrinogen-binding integrin subunits (αβ and β3), we did not see an effect of these subunits on fibrin structure. This lack of integrin effect contrasts that previously seen on platelets and HUVECs, but may reflect decreased relative density of integrins on the surface of fibroblasts versus other cells. We observed a minor effect of the cells on fibrin structure in clots formed by exogenous thrombin addition that was not blocked by RGDS-containing peptides. Besides integrins, vascular endothelial-cadherin and intercellular adhesion molecule-1 (ICAM-1) also bind fibrin(ogen). Preliminary experiments demonstrate that ICAM-1-blocking antibodies did not alter fibrin structure, suggesting that ICAM-1 does not influence fibrin structure on these cells. Further studies are ongoing to identify the nature of these interactions.

Cellular procoagulant activity is determined by expression of pro- and anticoagulant molecules. For example, cells expressing high levels of tissue factor and phosphatidylserine support higher rates of thrombin generation. Conversely, expression of tissue factor pathway inhibitor and thrombomodulin downregulate thrombin generation. Cells also up- and downregulate fibrinolysis via release of tPA and plasminogen activator inhibitor (PAI)-1, respectively, which may be expected to modulate lysis of fibrin proximal to the cell, before affecting fibrin distal to the cell. As with pro- and anticoagulant activities, expression of these pro- and antifibrinolytic activities are unique to different cells and vascular beds. Moreover, pathological triggers (eg, infection, inflammation, stasis) differentially modulate the expression of these activities on different cell types. Given our findings, it is likely that different cell types in different vascular beds would uniquely and specifically regulate procoagulant and fibrinolytic activities after vascular injury or pathological insult, and therefore the formation, structure, and stability of fibrin. The net influence of these cellular differences on fibrin quality may account, at least in part, for vascular bed–specific fibrin deposition observed in various studies.
A growing number of studies suggest that abnormal fibrin structure contributes to an individual’s risk of arterial thrombosis.\(^1\)\(^-\)\(^3\) We have previously shown that elevated prothrombin levels produce clots composed of an abnormally dense network composed of thin fibrin fibers,\(^4\) suggesting that abnormal fibrin structure contributes to the increased risk of venous thromboembolism in patients with the G20210A mutation.\(^8\)\(^-\)\(^9\) It has also been hypothesized that abnormal clot quality contributes to the risk of pulmonary embolism after deep vein thrombosis.\(^4\) Our study demonstrated fibrinolysis distal to the cell surface before proximal to the surface, consistent with processes thought to occur under normal conditions in vivo. However, structurally inadequate regions within a clot, poor-anchoring of intravascular clots to the vessel wall, or increased cell-derived fibrinolytic activity that triggers premature lysis of fibrin proximal to the cell surface may promote embolization in vivo. Further studies are warranted to understand the relationship between fibrin structure, stability, and embolization.

In summary, our findings demonstrate that cellular procoagulant activity dictates clot structure and stability as functions of not only the rate of thrombin generation, but also the three-dimensional location of fibrin within the clot. Specifically, during in situ thrombin generation, fibrin forms more quickly, assumes a denser network, and is relatively resistant to lysis proximal to the procoagulant cell surface versus distal. This morphology can arise independently of direct interactions between cellular receptors and the fibrin network, but may be further modulated by cellular receptors on certain cells. This study is a first step in understanding how cells contribute to fibrin formation. Identifying specific cellular influences on fibrin structure is essential for determining antithrombotic targets for preventing thrombus formation or stabilizing fibrin clots to prevent embolism.

**Acknowledgments**

The authors thank Drs Susan Lord and Oleg Gorkun for their thoughtful contributions and Victoria Madden, Laura Gray, and Brittany Larson for their excellent technical assistance.

**Sources of Funding**

This study was supported by NIH grants K01 AR051021 (to A.S.W.) and T32 ES007017 (to R.A.C.), and the Gustavus and Louise Pfeiffer Research Foundation (to A.S.W.).

**Disclosures**

A.S.W. has received research funding from Novo Nordisk Inc within the last two years.

**References**


Cellular Procoagulant Activity Dictates Clot Structure and Stability as a Function of Distance From the Cell Surface
Robert A. Campbell, Katherine A. Overmyer, C. Robert Bagnell and Alisa S. Wolberg

Arterioscler Thromb Vasc Biol. published online October 30, 2008;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 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/early/2008/10/30/ATVBAHA.108.176008.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2008/10/31/ATVBAHA.108.176008.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/
CELLULAR PROCOAGULANT ACTIVITY DICTATES CLOT STRUCTURE AND STABILITY AS A FUNCTION OF DISTANCE FROM THE CELL SURFACE

Campbell, Robert A. Katherine A. Overmyer, C. Robert Bagnell, Alisa S. Wolberg

EXPANDED METHODS FOR ONLINE PUBLICATION

Proteins and Materials
Dulbecco’s modified Eagle’s Medium (DMEM) with high glucose and L-glutamine, 0.05 % Trypsin/EDTA, and phosphate buffered saline (PBS) were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT). Chromozym Th (tosyl-gly-pro-arg-pNA) was from Boehringer-Mannheim (Indianapolis, IN). Synthetic peptide arg-gly-asp-ser (RGDS) and goat anti-rabbit peroxidase conjugate antibody were from Calbiochem (La Jolla, CA). Prothrombin was generously provided by Dr. Dougald M. Monroe (University of North Carolina at Chapel Hill). Thrombin, plasmin, corn trypsin inhibitor and factors Xa and Va were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Plasminogen- and fibronectin-free fibrinogen was purchased from Enzyme Research Laboratories (South Bend, IN). Immortalized human fibroblasts (NHF1-hTert) were provided by Dr. Marila Cordeiro-Stone (University of North Carolina at Chapel Hill).

Preparation of AlexaFluor-488-labeled fibrinogen
AlexaFluor-488-labeled fibrinogen was prepared according to the manufacturer’s instructions [Invitrogen Corporation (Carlsbad, CA)]. Labeled fibrinogen contained ~8 molecules of dye/fibrinogen molecule. The preparation was frozen in aliquots that were thawed only once before each experiment. Turbidity measurements on fibrin formed in the presence or absence of labeled fibrinogen demonstrated that the inclusion of labeled fibrinogen did not affect polymerization (data not shown).

Cell Culture
Immortalized human fibroblasts (NHF1-hTert) were grown in DMEM with 2 mM glutamine and 10% FBS at 37 °C in a humidified atmosphere of 5% CO2. Cultures were used at 80-95% confluence between passages 16-35.

Preparation of platelet-free plasma (PFP)
Blood was collected with informed consent under a protocol approved by the University of North Carolina Institutional Review Board. Whole blood was drawn into 3.2% citrated saline and corn trypsin inhibitor (18.5 μg/mL, final) and centrifuged at 150xg for 10 minutes. The upper layer (platelet-rich plasma) was centrifuged at 13000xg for 10 minutes to make platelet-free plasma. Platelet-free plasma from 16 individuals with normal activated partial thromboplastin times were pooled to yield normal pooled platelet-free plasma (PFP).

Western blot for integrin αv and β3 subunits
Cell lysates were prepared as described [Nan B, Lin P, Lumsden AB, Yao Q, Chen C. Effects of TNF-alpha and curcumin on the expression of thrombomodulin and endothelial protein C receptor in human endothelial cells. *Thromb Res.* 2005;115:417-426]. Thirty μg of total protein was separated by 10% SDS-PAGE. Integrin subunits were detected with rabbit anti-human αv or β3, generously provided by Dr. Susan S. Smyth (University of Kentucky), and goat anti-rabbit peroxidase-conjugated antibody. Proteins were visualized with enhanced chemiluminescence detection (Amersham Biosciences, Piscataway, NJ).
**Conditions for Laser Scanning Confocal Microscopy**

Clots were scanned with a Zeiss LSM5 Pascal laser scanning confocal microscope (Carl Zeiss, Inc) linked to a Zeiss Axiovert 200M microscope equipped with a Zeiss 63x 1.4 NA oil immersion plan apo-chromatic lens. The 488 nm line of a medium power multi-line argon ion laser was used for excitation and a 505-530-nm band-pass filter for emission. A computer equipped with Carl Zeiss software (v1.5) was used to operate the system. Optical sectioning was achieved by closing the pinhole in the front of the detector to one airy unit. The zoom factor was 1. Thirty optical sections (1024 x 1024 pixels each) in three randomly-chosen locations were collected at 0.36-µm intervals in the z-axis at the cell surface (defined as when the cells were in focus using differential interference contrast microscopy) and 40-µm above the cell surface, thus covering a region 10.8-µm thick at and above the cell surface. Image volumes were 146x146x10 µm. Single images were collected in 15.47 seconds. Optical resolution was ~0.14 µm in the xy-plane and ~0.5 µm on the z-axis. The sectioning interval in z was smaller than the calculated z-axis optical section resolution to achieve Nyquest sampling in z based on the Zeiss software calculation. No correction was made for refractive index mismatch.

**Analysis of LSCM Images**

Images were deconvolved using 3D deconvolution algorithms in AutoQuant’s Autodeblur version x1.4.1 (Media Cybernetics Inc., Bethesda, MD). Fibrin network density and diameter were analyzed using Image J (version 1.37v, National Institutes of Health) by placing a random grid of 2 pixel crosses on individual slices (~121-144 crosses/slice). Network density was determined by counting fibers intersecting the middle of the crosses, divided by the total number of crosses. Crosses placed over cells were subtracted from the total number of crosses. Fiber thickness was measured on fibers intersecting a cross (~20 fibers/slice). For lysis experiments, images were deconvolved using 2D deconvolution algorithms in AutoQuant’s Autodeblur. Fibers were counted using the angiogenesis/endothelial tube formation application module in MetaMorph version 7.1.3.0 (Molecular Devices, Dowington, PA).

**Structural Analysis by TEM**

Clots were fixed overnight in 4% paraformaldehyde/0.15% glutaraldehyde in PBS. Fixed clots were removed from the wells, washed, embedded in LR white, and cross-sectioned (90 nm) to visualize cells and fibrin. Post-embedding immunogold labeling was performed by adding rabbit anti-human fibrinogen antibody (Dako Corporation, Carpinteria, CA) followed by goat anti-rabbit 20-nm gold-conjugated antibody (BBInternational, Lianishan, Cardiff) to confirm the identity of fibrin fibers in certain experiments. Clots were imaged using a LEO EM910 transmission electron microscope (Carl Zeiss SMT, Peabody, MA) operating at 80kv. Digital images were recorded using a Gatan Orius CCD Digital Camera and Digital Micrograph 3.11.0 (Gatan INC, Pleasanton, CA).
LEGENDS FOR SUPPLEMENTAL FIGURES

Figure S1. Varying the prothrombin concentration affects thrombin generation and clot formation on human fibroblasts. Factors Xa, Va, CaCl2 and 0.014 prothrombin (circles) or 1.4 µm prothrombin (squares) were incubated with confluent fibroblasts in the presence or absence of fibrinogen, as described in the Methods. A) Thrombin generation. B) Clot formation. The inset in panel B shows an enlargement of the first 2 minutes of the clotting reaction. The data (± standard deviation) shown are from one experiment, representative of three independent experiments.

Figure S2. Fiber diameter is not influenced by distance from the cell surface. Fibrin fiber diameter (± standard error) was determined as described in Methods in the presence of cells with 0.014 µm prothrombin (circles), 1.4 µm prothrombin (squares), or 2 nM thrombin (diamonds).

Figure S3. Fibrin density is determined by the distance from the cell surface and is not altered by the presence or absence of RGDS peptide. Fibrin network density (± standard error) was determined as described in Methods in the absence and presence (open and closed symbols, respectively) of clots formed over fibroblasts with A) 0.014 µM prothrombin, B) 1.4 µM prothrombin, or C) 2 nM thrombin.

Figure S4. Clot formation and lysis are determined by the distance from the cell surface. Reactions were performed by combining factors Xa, Va, prothrombin, fibrinogen, and CaCl2 in the presence of plasmin (0.024 µM, final), as described in the Methods. Movies demonstrate fibrin formation and subsequent lysis at (A and C) and 50 µm above (B and D) the cell surface in the presence of 0.014 (A and B) and 1.4 µM (C and D) prothrombin. For all movies, the frame size is 146 µm x 146 µm and the time points are 15 seconds apart.
Figure S1.

A.

B.
Figure S2.
Figure S3.