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# Cellular procoagulant activity dictates clot structure and stability as a function of distance from the cell surface

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## Abstract

**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—**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.

**Results:** *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 – 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 clot's structural heterogeneity 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.

### Keywords

coagulation; fibrin clot structure; thrombin generation; fibrinolysis

Following 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

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fibrin fibers, while high thrombin concentrations produce clots composed of a dense network of thin fibers. Additional studies have correlated fibrin architecture with a clot's resistance to mechanical and fibrinolytic disruption.[8–11] 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 performed 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. Fibroblasts were washed with PBS and immediately incubated with factors Xa, Va, and $CaCl_2$ (1 nM, 5 nM, 5 mM, final, respectively) and prothrombin (0.014 μM or 1.4 μM, final, as indicated) in HBS. Thrombin generation was measured by quenching 10 μL aliquots of supernatant with 90 μL of 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (pH 7.4), 150 mM NaCl (HBS) containing 10 mM ethylenediamine tetraacetic acid (EDTA) and 1 mg/mL bovine serum albumin (BSA). Quenched reactions were diluted 1:10 in HBS containing 1 mM EDTA and 0.5 mM Chromozym Th. The substrate cleavage rate (change in absorbance at 405 nm *versus* time) was measured using a SpectraMax 340PC microplate reader (Molecular Devices, Sunnyvale, CA).[15] Clot formation was monitored at 405 nm in parallel experiments that included 2 mg/mL fibrinogen.[8, 13–15] In reactions in which clot formation was initiated by exogenous thrombin addition, fibrinogen, $CaCl_2$, and 2 nM thrombin (final) were pre-mixed and immediately added to the cells.

### Structural Analysis by Laser Scanning Confocal Microscopy (LSCM)

Cells were grown to confluence on LabTek II Chamber uncoated #1.5 cover-glasses (Nalge Nunc International, Naperville, IL). 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, St. Louis,

MO) to eliminate surface effects of glass on the polymerizing fibrin.[16] For lysis studies, cells were grown to confluence on MatTek uncoated #1.5 glass 35 mm dishes (Ashland, MA). Clots were formed as above in the presence of 0.024 μM 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 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 Thermanox tissue culture cover slips (Miles Laboratories, Naperville, IL) or in eight well Permanox chamber slides (Nalge Nunc International, Rochester, NY) for transmission electron microscopy (TEM). Cells were washed with PBS and clots were formed by incubating fibroblasts with re-calcified (10 mM, final) platelet-free plasma 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's 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 *vs*. 0.014 μM prothrombin ($p < 0.007$) (Table I, Figure S1A). To determine the effect of 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 μM prothrombin) exhibited a shortened onset and 2.2-fold increased polymerization rate compared to clot formation during low thrombin generation rates (Table I, Figure S1B). 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 I, Figure S1B). Such a non-linear relationship between fibrin polymerization rate and final turbidity has been observed in previous studies performed in the presence and absence of cells.[5–9, 14, 15, 17] 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 inter-clot differences in structure as a function of prothrombinase activity, as well as intra-clot 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 Figures 1a, 1d with 1b, 1e, respectively, Table II).

Remarkably, however, the network density was ~25% higher in regions proximal (0 – 10 μm) *versus* distal to (40 – 50 μm) the cell surface regardless of the thrombin generation rate (compare Figures 1a, 1b with 1d, 1e, respectively, Table II). In contrast, fiber thickness did not change as a function of distance from the cell surface (Table II). Of note, however, fiber diameter (200–400 nm) is near the lower resolution limit for LSCM (~200 nm)[5, 18], such that subtle changes may not be apparent. Nonetheless, the spatial-dependence of fibrin density in clots formed during *in situ* thrombin generation on the cell surface suggested that cells modulate the three-dimensional structure of the clot.

### *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 fibrin (ogen)[19–21], and that the αvβ3 andαIIbβ3 integrins can directly modulate fibrin structure on human umbilical vein endothelial cells (HUVEC) and platelets, respectively.[22–24] Since the fibroblasts used in our study expressed both αv and β3 subunits (data not shown)[19–21, 25], we examined the influence of αvβ3 and other RGD-binding integrins on fibrin architecture by comparing clots formed in the presence and absence of the integrin-blocking peptide, RGDS (500 μM, 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 – 10 μm) *versus* distal (40 – 50 μm) to the cell surface (Table II). The mean network density was identical with and without RGDS (compare Figures 1d and 1e with 1g and 1h, respectively, Table II), suggesting that neither αvβ3, αIIbβ3, 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 HUVEC[23] and platelets[24] demonstrated that integrin-mediated influences on fibrin structure are detectable following exogenous thrombin addition. Under these conditions, the mean network density in regions proximal (0–10 μm) and distal (40–50 μm) to the cell surface were indistinguishable (Figures 1c, 1f, Table II), and addition of RGDS peptide did not alter the fibrin appearance (compare Figures 1f and 1i, Table II). These findings suggest that *in situ* thrombin generation on the cell surface induces 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 (Figure S2), 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 μM and 1.4 μM 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, Table II), 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 (Figure S3). To confirm that the decrease was not due to non-specific 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 0–50 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

Since 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 μM prothrombin), fibrin formed proximal, but not distal to the cell surface within 50 seconds (Figure 3, Movies S4A, S4B). After two minutes, fibrin was present proximal and distal to the cell surface. Fibrin distal to (50 μm above) the cell surface lysed 328 ± 82 seconds prior to lysis at the cell surface. During high thrombin generation rates (1.4 μM prothrombin), we observed immediate fibrin formation near the cell surface < 15 seconds after clotting was initiated (Figure 3, Movies S4C, S4D). As in reactions with low thrombin generation, fibrin appearance proximal to the surface preceded that distal to (50 μm above) the cell surface, and fibrin lysed above the cell surface 449 ± 47 seconds prior to lysis near the cell surface. Interestingly, the lysis rate (decrease in fiber number/$\mu m^2$ *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 slowly than those in a loose network.[9]

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 due 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 4, plasma clots exhibited denser fibrin network near the fibroblast surface *versus* 50 μm above the cell surface. The denser network was not abolished by addition of RGDS peptide, suggesting RGDS-binding integrins did not mediate this interaction. TEM confirmed these findings, demonstrating 4-fold more fibers proximal (~1 μm) *versus* distal to (~5 μm away) the cell surface (Figure 4c–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 non-uniform, with extrinsic and intrinsic activities differentially modulating the initiation and propagation of fibrin formation in space.[12] Second, the concentration of thrombin present during fibrin gelation dictates the architecture of the fibrin clot.[5–9] Third, the fibrin clot quality influences its susceptibility to fibrinolysis.[8–11] 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 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-heterogenous morphology is likely due 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)[27] Additionally, both mathematical simulations and *in vitro* studies predict that the diffusional limit of procoagulant enzymes through the fibrin/platelet layer limits thrombus height.[28, 29] 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.[29] 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 (αv 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 HUVEC[22–24], 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 RGD-containing peptides. Besides integrins, vascular endothelial-cadherin[30] and intercellular adhesion molecule-1 (ICAM-1)[21, 31] also bind fibrin(ogen). Preliminary experiments demonstrate that ICAM-1-blocking antibodies do 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 anti-coagulant molecules. For example, cells expressing high levels of tissue factor and phosphatidylserine support higher rates of thrombin generation.[12] Conversely, expression of tissue factor pathway inhibitor and thrombomodulin down-regulate thrombin generation.[32, 33] Cells also up- and down-regulate fibrinolysis via release of tPA and PAI-1, respectively [34], which may be expected to modulate lysis of fibrin proximal to the cell, prior to affecting fibrin distal to the cell. As with pro- and anti-coagulant activities, expression of these pro- and anti-fibrinolytic activities are unique to different cells and vascular beds.[35] Moreover, pathologic triggers (*e.g.*, infection, inflammation, stasis) differently modulate the expression of these activities on different cell types.[35] Given our findings, it is likely that different cell types in different vascular

beds would uniquely and specifically regulate procoagulant and fibrinolytic activities following vascular injury or pathologic 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.[36–38]

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[14], suggesting that abnormal fibrin structure contributes to the increased risk of venous thromboembolism in patients with the G20210A mutation.[39] It has also been hypothesized that abnormal clot quality contributes to the risk of pulmonary embolism following deep vein thrombosis.[4] Our study demonstrated fibrinolysis distal to the cell surface prior to 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 for the first time 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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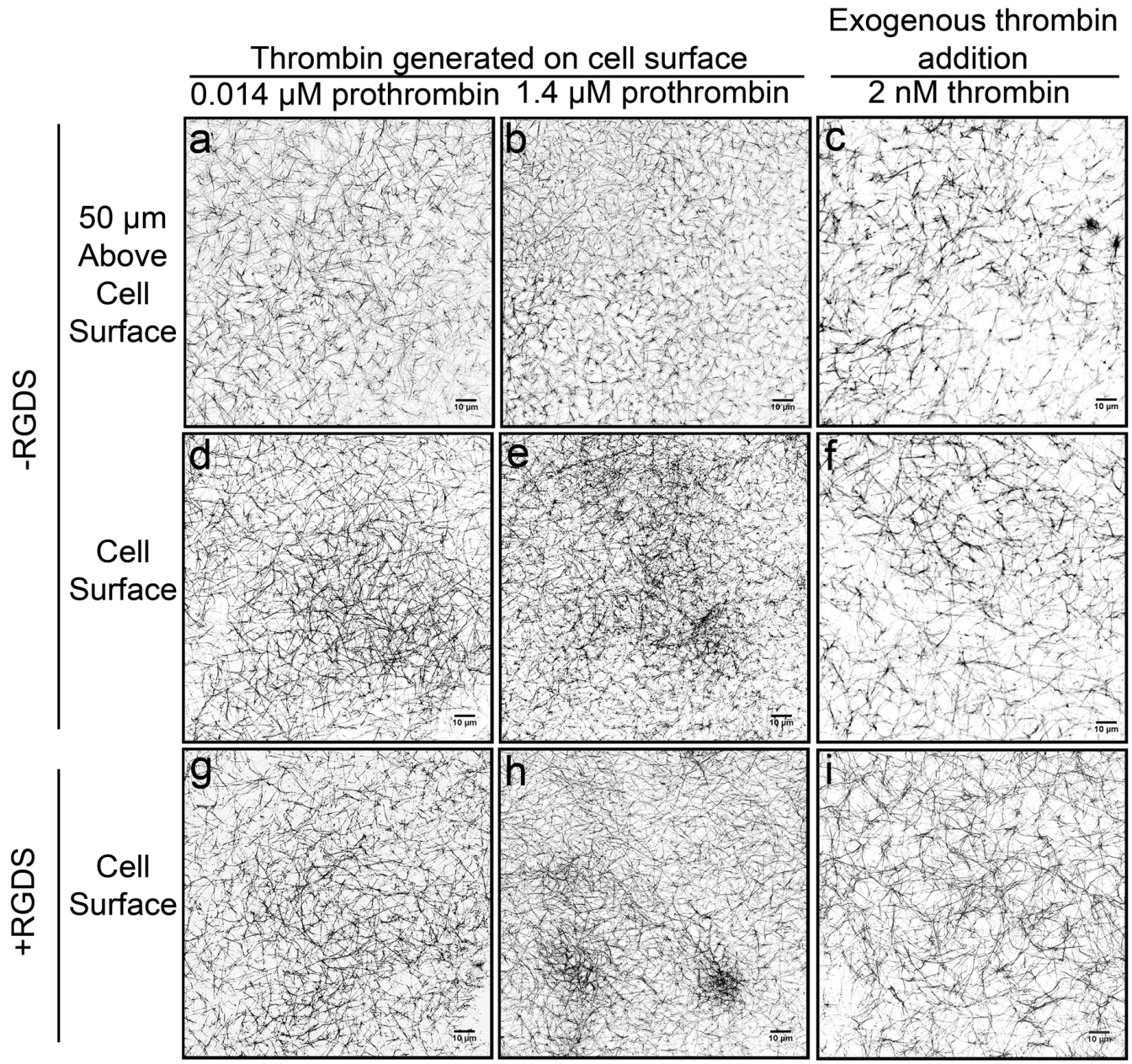
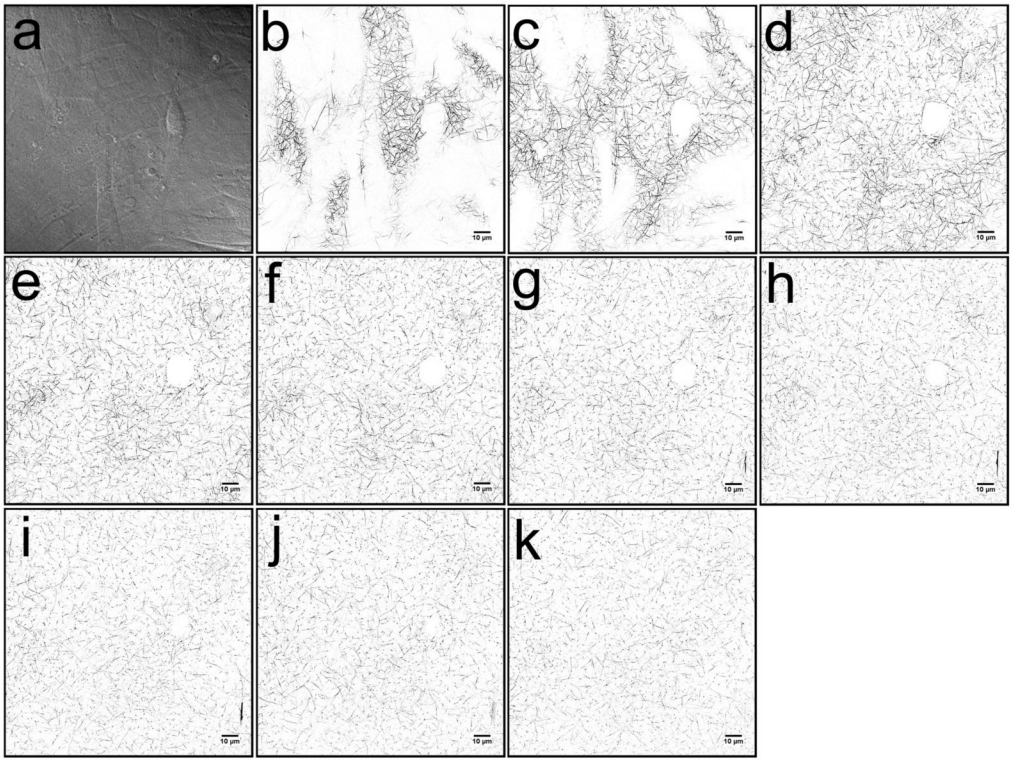



**Figure 1. *In situ* thrombin generation on the cell surface modulates clot structure in three dimensions**

Clots were formed as described in the Methods in the absence (a–f) or presence (g–i) of RGDS peptide. Three-dimensional projections show clot architecture in 10-μm sections at (0–10 μm) and above (40–50 μm) the cell surface. Each image is from one experiment, representative of 3–6 independent experiments. Darker areas show increased fibrin density. Bar = 10 μm.



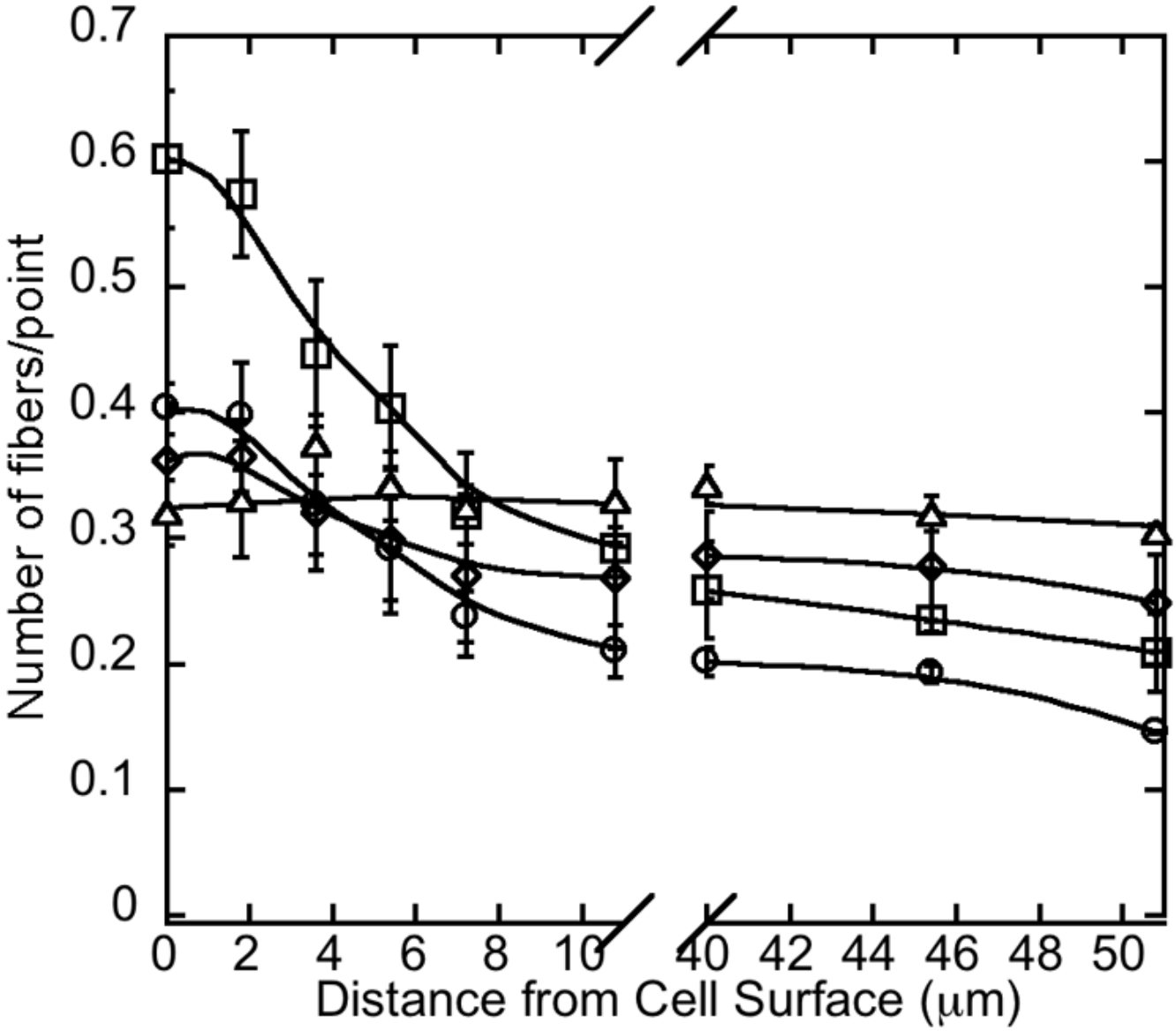
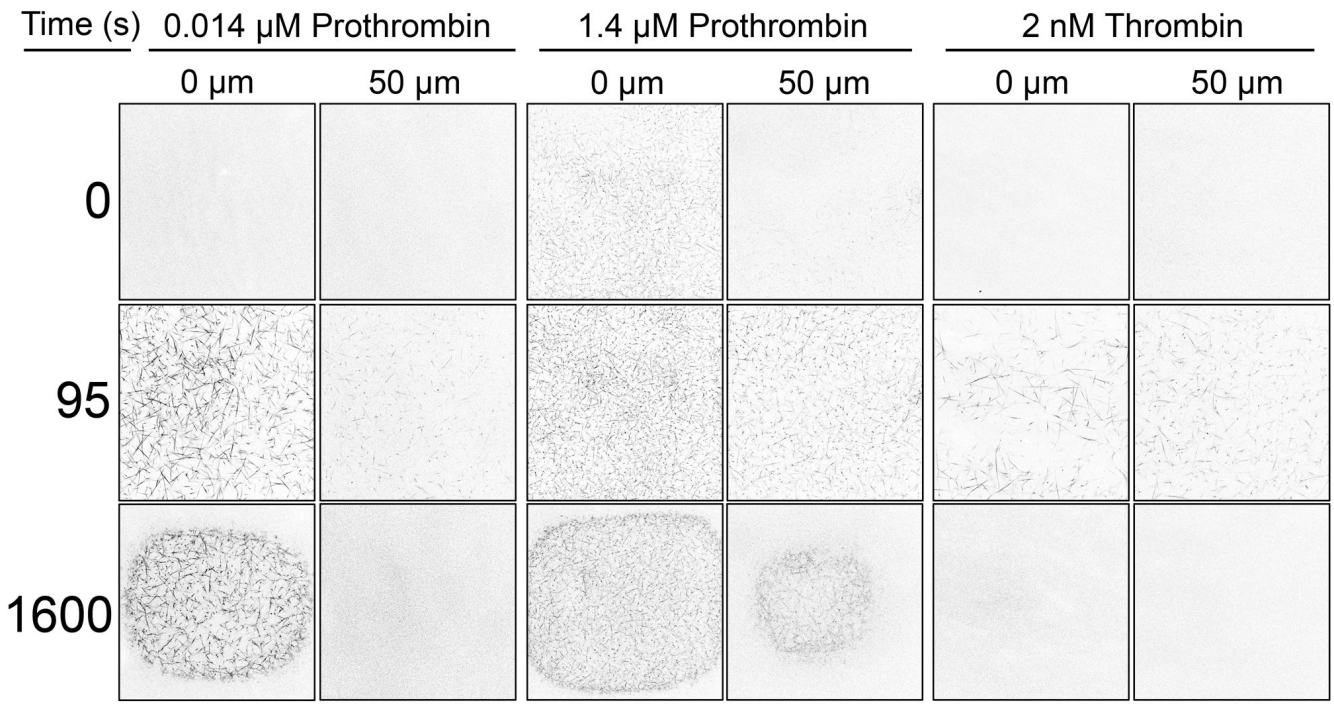
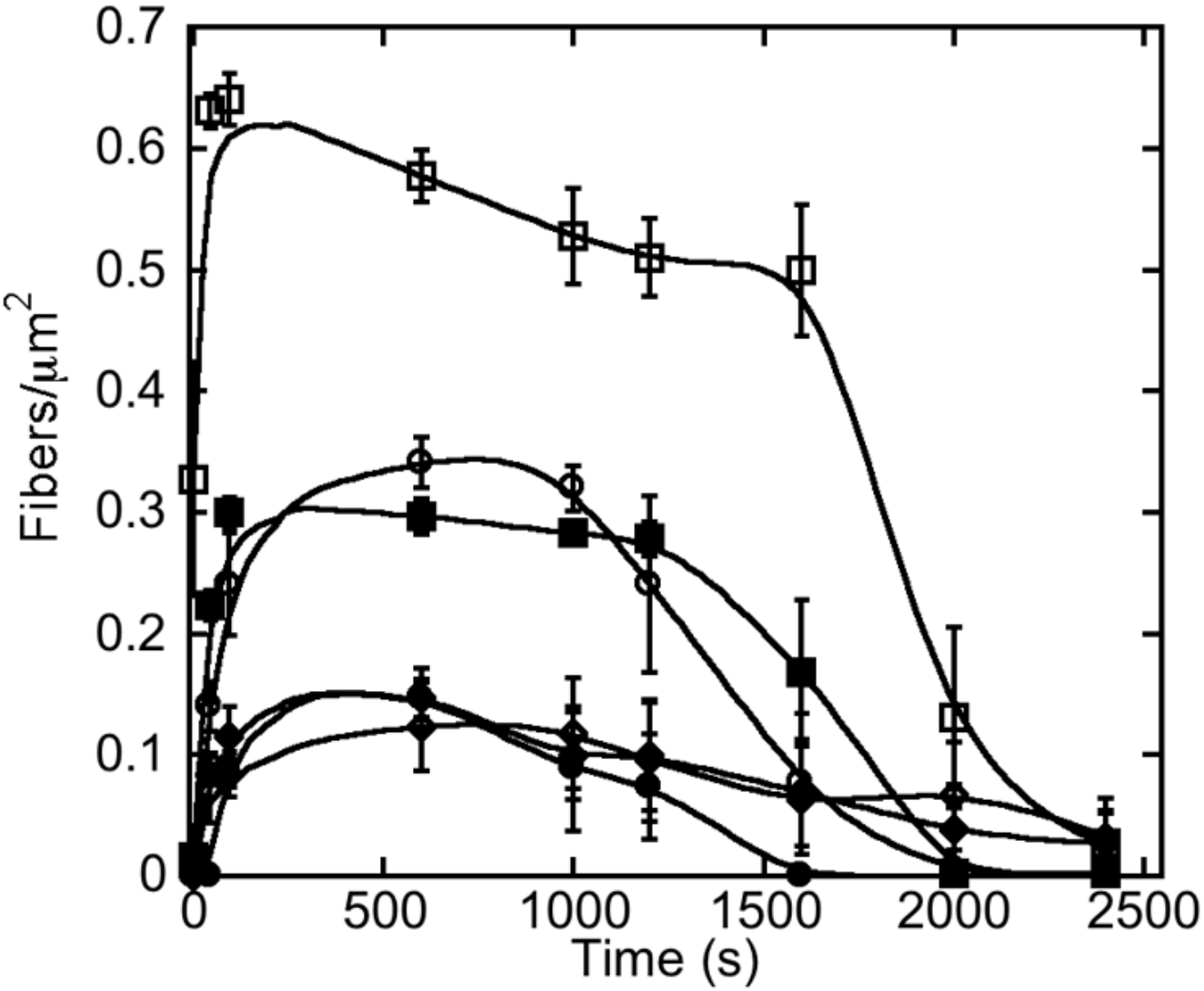
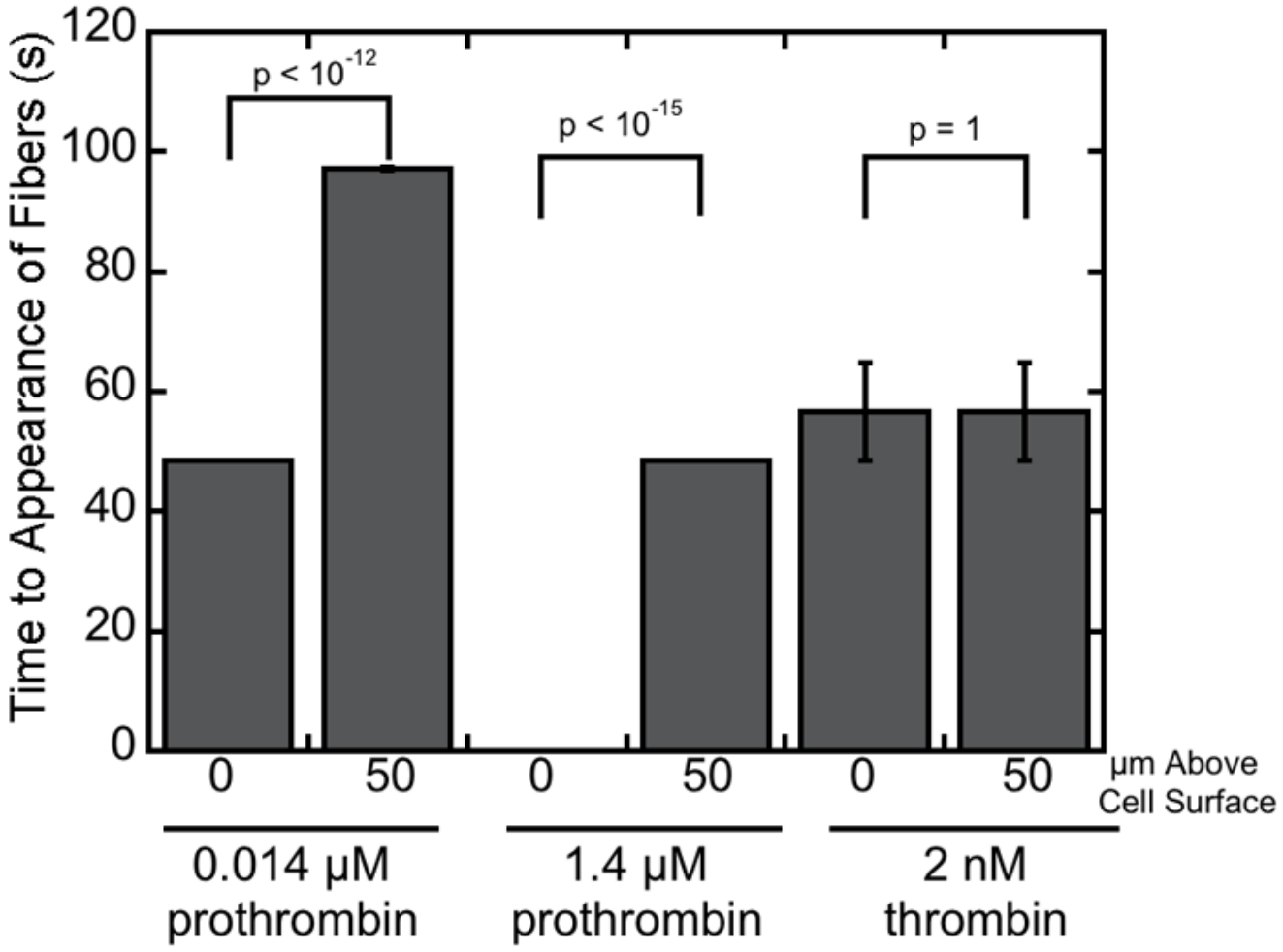



**Figure 2. Fibrin structure is determined by the distance from the cell surface**

A) Images from one experiment in which clots were formed by combining factors Xa, Va, II, fibrinogen, and $CaCl_2$ (1 nM, 5 nM, 1.4 μM, 2 mg/mL, and 5 mM, respectively) as described in the Methods. a) Differential interference contrast image. b – k) Individuals slices every 1.08 μm in the z-plane: b) 0, c) 1.08, d) 2.16, e) 3.24, f) 4.32, g) 5.4, h) 6.48, i) 7.56, j) 8.64, and k) 9.72 μm from the cell surface. Darker areas show increased fibrin density. Bar = 10 μm. B) Fibrin network density (± standard error) was determined as described in Methods in the presence of cells with 0.014 μM prothrombin (circles), 1.4 μM prothrombin (squares), 2 nM thrombin (diamonds), or in the absence of cells with 2 nM thrombin (triangles).







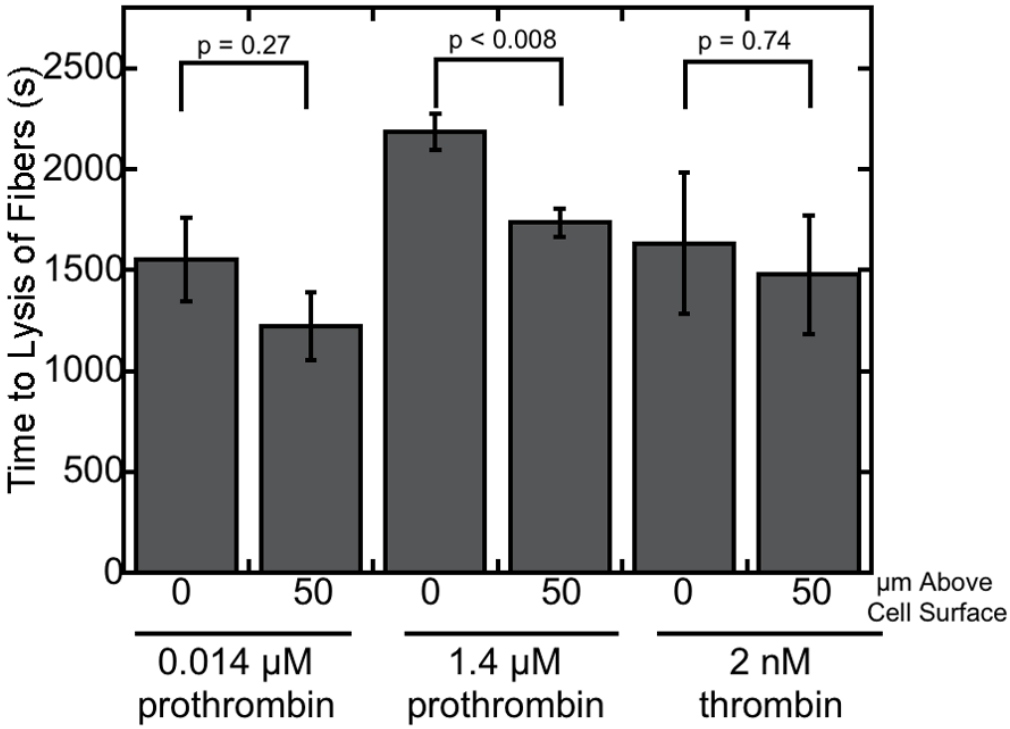
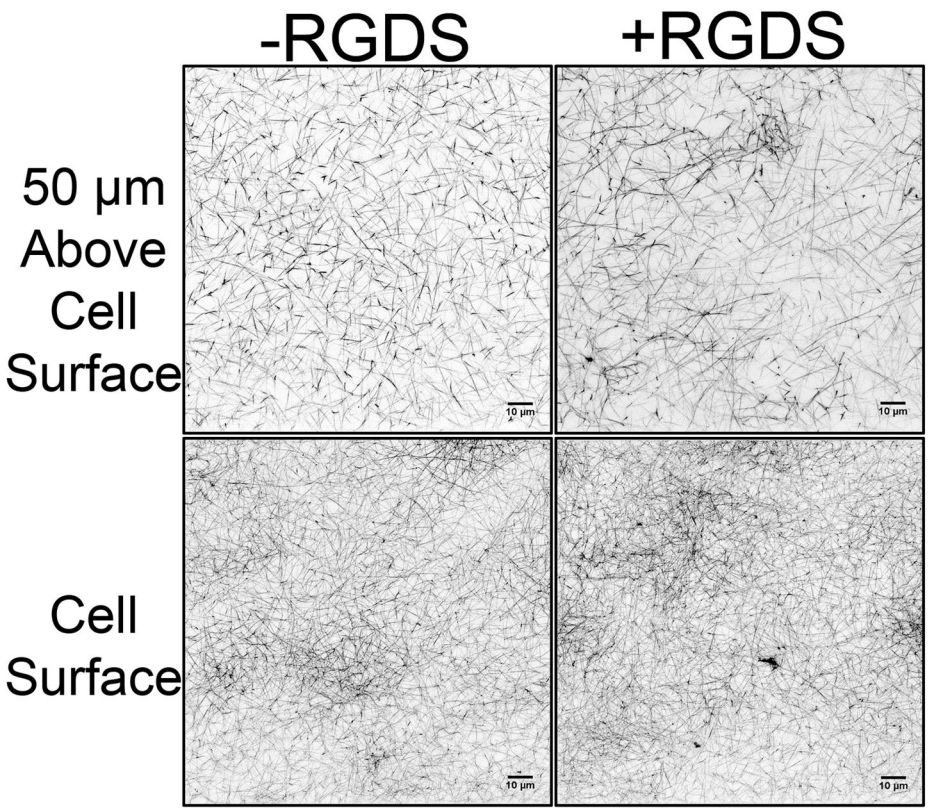
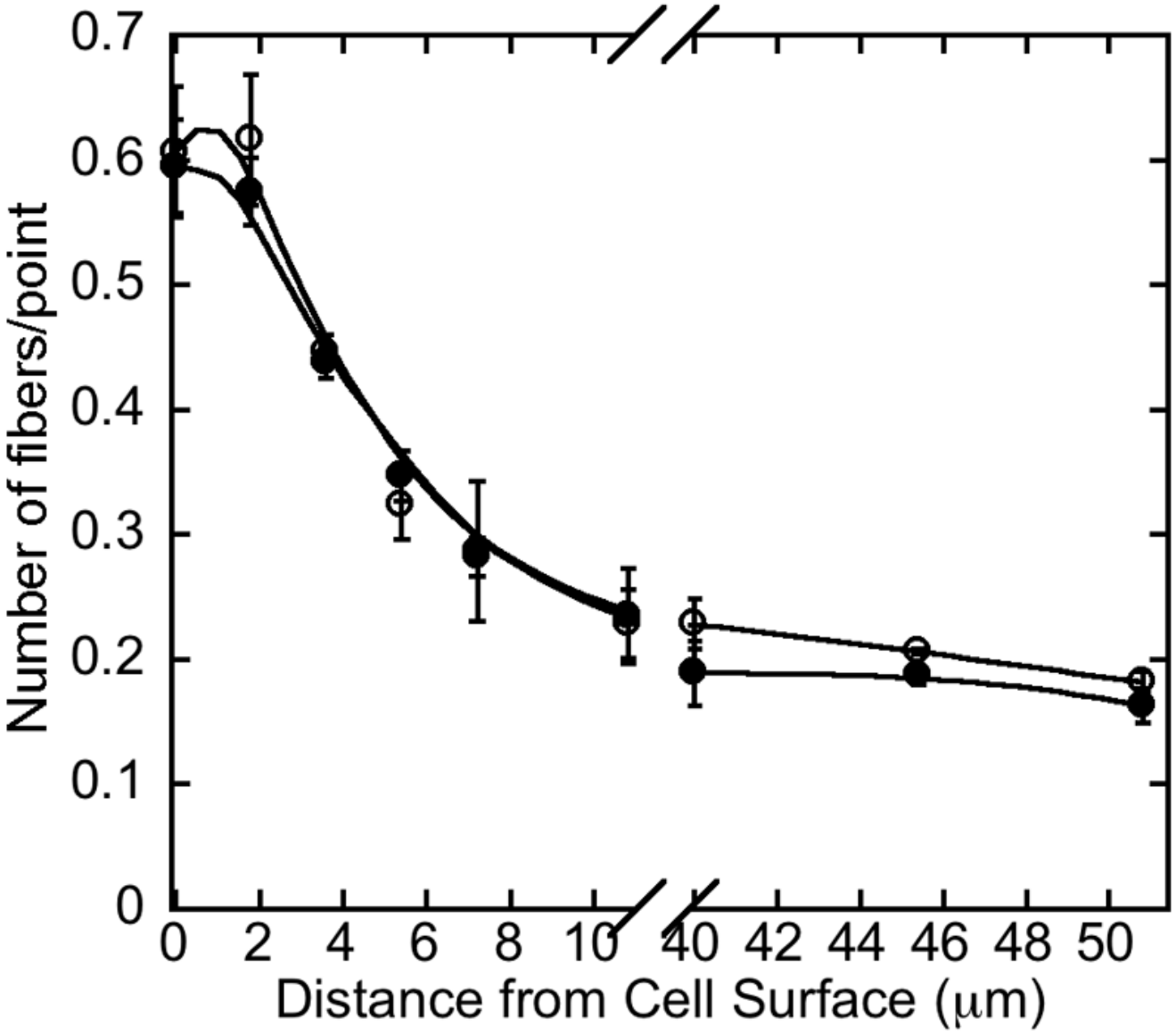
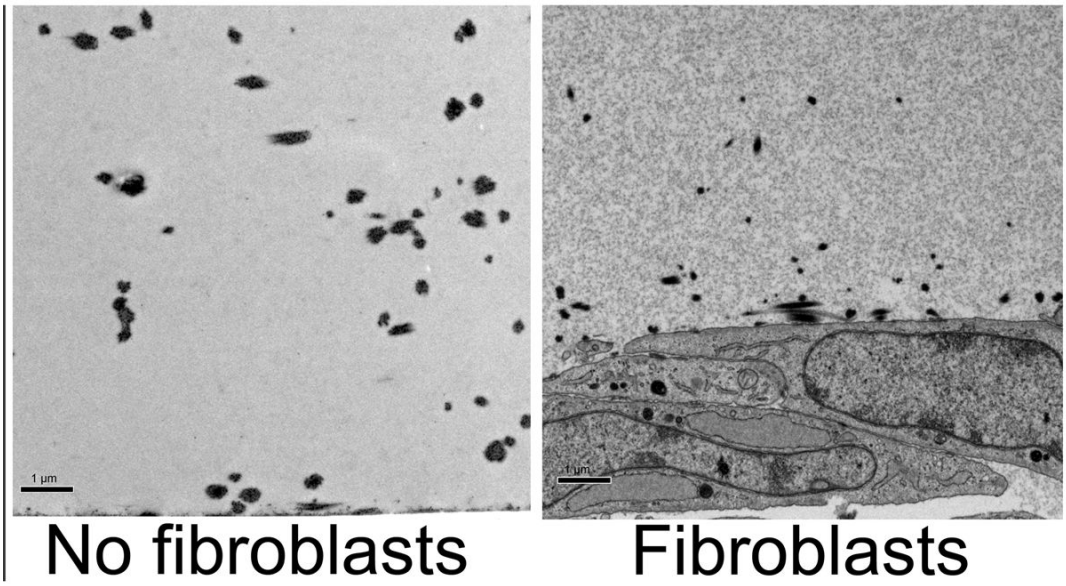



**Figure 3. Clot formation and lysis are determined by the distance from the cell surface**

Factors Xa, Va, prothrombin, fibrinogen, and $CaCl_2$ were combined in the presence of plasmin, as described in Methods. A) Images are from one experiment, representative of four independent experiments. Images are the same size as in figure 1. The circular lysis pattern reflects the distribution of light power across the lens (30% greater in the center than at the periphery at 63× oil objective). B) Change in the fiber number over time (± standard error, n = 4 – 6) in clots formed over cells with 0.014 μM prothrombin (circles), 1.4 μM prothrombin (squares), or 2 nM thrombin (diamonds), at (open symbols) and above (closed symbols) the cell surface. C) Fiber formation was defined as the first appearance of fibers in the field of view. D) Fiber lysis was defined as the disappearance of all fibers in the field of view.







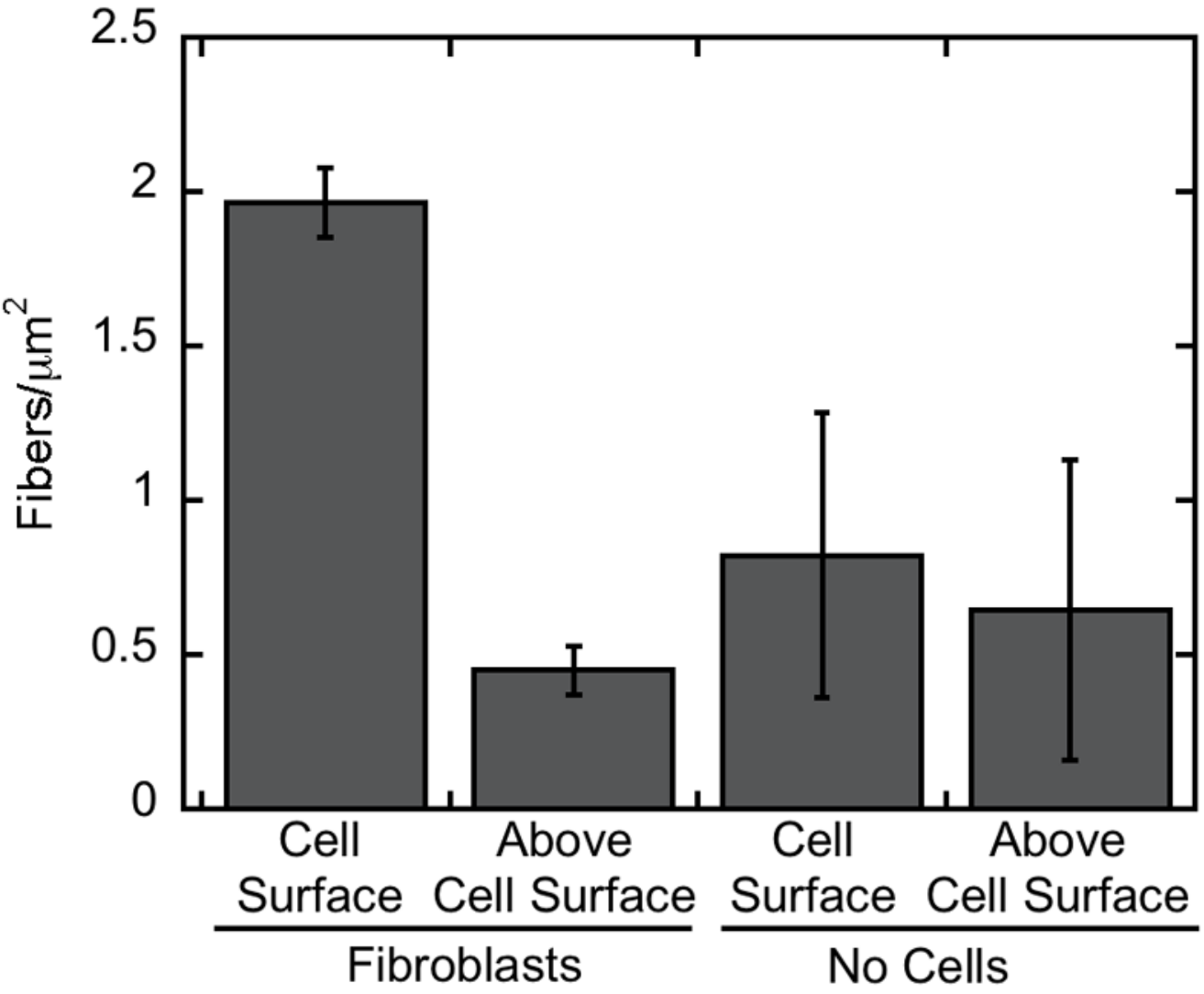



**Figure 4. Cells modulate the network density of plasma clots**

Re-calcified PFP was incubated with or without fibroblasts, as described in the Methods. A) Three-dimensional projections show clot architecture in 10 μm sections at (0–10 μm) and above (40–50 μm) the cell surface. Each image is from one experiment, representative of 2 independent experiments. Bar = 10 μm. B) Fibrin network density (± standard deviation) in the absence and presence (open and closed symbols, respectively) of 500 μM RGDS. C) TEMs of fibrin fibers (black dots) in clots formed in the presence or absence of fibroblasts. In both images, the bottom of the reaction chamber is oriented at the bottom of the image. Bar = 1 μm. D) Fibrin fibers were counted on micrographs recorded at 6300X by placing a 1-μm thick box parallel to the cell surface 0.25 μm and 5 μm from the cell surface and counting fibers inside the box.

## TABLE I

Properties of Clots Formed by *in situ* Thrombin Generation

| | Thrombin Generation Rate (nM/min) | Clotting Onset (seconds) | Fibrin Polymerization Rate (mOD/min) | Final Turbidity at 405 nm |
|---|---|---|---|---|
| 0.014 μM | 0.402 ± 0.11 | 77 ± 5.0 | 77 ± 7.2 | 0.589 ± 0.12 |
| 1.4 μM | 16.3 ± 5.44 | 33 ± 14.6 | 168 ± 20.3 | 0.536 ± 0.11 |
| P | <0.007 | < 0.008 | < 0.002 | NS |

**TABLE II**

Fibrin Structure in 10 μm sections 0–10 μm and 40–50 μm from the Cell Surface

| | Density (0 – 10 μm) | Density (40 – 50 μm) | P (0–10 μm vs. 40–50 μm) | Fiber Diameter (0 – 10 μm) | Fiber Diameter (40 – 50 μm) | P (0–10 μm vs. 40–50 μm) |
|---|---|---|---|---|---|---|
| 0.014 μM | 0.763 ± 0.025 | 0.568 ± 0.048 | < 0.008 | 0.300 ± 0.024 | 0.311 ± 0.032 | NS |
| 0.014 μM + RGDS | 0.720 ± 0.027 | 0.569 ± 0.020 | < 0.002 | ND | ND | - |
| P (± RGDS) | NS | NS | - | - | - | - |
| 1.4 μM | 0.856 ± 0.008 | 0.662 ± 0.052 | < 0.02 | 0.263 ± 0.002 | 0.268 ± 0.007 | NS |
| 1.4 μM + RGDS | 0.866 ± 0.042 | 0.607 ± 0.054 | < 0.003 | ND | ND | |
| P (± RGDS) | NS | NS | - | - | - | - |
| 2 nM thrombin | 0.715 ± 0.037 | 0.700 ± 0.060 | NS | 0.313 ± 0.013 | 0.311 ± 0.014 | NS |
| 2 nM thrombin + RGDS | 0.683 ± 0.012 | 0.663 ± 0.040 | NS | ND | ND | - |
| P (± RGDS) | NS | NS | - | - | - | - |