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Fibrinogen counteracts the antiadhesive effect of fibrin-bound plasminogen by preventing its activation by adherent U937 monocytic cells

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Summary

Background—Fibrinogen and plasminogen strongly reduce adhesion of leukocytes and platelets to fibrin clots, highlighting a possible role for these plasma proteins in surface-mediated control of thrombus growth and stability. In particular, adsorption of fibrinogen on fibrin clots renders their surfaces non-adhesive, while the conversion of surface-bound plasminogen to plasmin by transiently adherent blood cells results in degradation of a superficial fibrin layer, leading to cell detachment. Although the mechanisms whereby these proteins exert their antiadhesive effects are different, the outcome is the same: the formation of a mechanically unstable surface that does not allow firm cell attachment.

Objectives—Since fibrin clots in circulation are exposed to both fibrinogen and plasminogen, their combined effect on adhesion of monocytic cells was examined.

Methods—Fibrin gels were coated with plasminogen and its activation by adherent U937 monocytic cells in the presence of increasing concentrations of fibrinogen was examined by either measuring ¹²⁵I-labeled fibrin degradation products or plasmin amidolytic activity.

Results—Unexpectedly, the antiadhesive effects of two fibrin binding proteins were not additive; in fact, in the presence of fibrinogen, the effect of plasminogen was strongly reduced. An investigation of the underlying mechanism revealed that fibrinogen prevented activation of fibrin-bound plasminogen by cells. Confocal microscopy showed that fibrinogen accumulates in a thin superficial layer of a clot, where it exerts its blocking effect on activation of plasminogen.

Conclusion—The results point to a complex interplay between the fibrinogen- and plasminogen-dependent antiadhesive systems, which may contribute to the mechanisms that control the adhesiveness of a fibrin shell on the surface of hemostatic thrombi.

Keywords

adhesion; fibrinogen; fibrinolysis; thrombus; plasminogen

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Introduction

The formation of a hemostatic thrombus during blood vessel injury is a highly regulated event. The process ensures that a blood clot is sufficiently strong to seal the breach and prevent the loss of blood, but is not overly robust to cause vessel occlusion and thrombosis. Thrombus formation is triggered by platelet adhesion to the exposed subendothelial matrix and ensued by platelet activation and aggregation. The initial platelet accumulation is followed by thrombin-mediated generation of fibrin that stabilizes the entire thrombus. While platelet accumulation ceases shortly after the injury, fibrin deposition continues for several hours, resulting in the formation of an extensive fibrin network [1–4]. The presence of a fibrin cap on the surface of experimental thrombi has been recently documented using *in vivo* imaging [5–7]. It has also been shown that the surface of late thrombi contains very few adherent platelets and leukocytes [3,8–10]. The latter finding contrasts with numerous *in vitro* observations in which fibrin was found to be highly adhesive for these blood cells [11–13]. However, it seems intuitive that fibrin deposited on the surface of newly formed hemostatic clots must not be adhesive because if not curtailed, uncontrollable platelet adhesion can readily lead to their activation and thrombus propagation. Likewise, adhesion of leukocytes, which are known to contain potent fibrinolytic enzymes, must be regulated to allow early hemostasis to proceed unchallenged until the growth and stability of the fibrin clot is established. Therefore, a proper balance between adhesive and antiadhesive mechanisms operating on the surface of fibrin clots in circulation may play an important role in the control of thrombus formation, stability and timely dissolution. In spite of the obviousness of this idea and some experimental evidence to support it, the fact that the surface of fibrin clots in circulation is not adhesive for blood cells is unappreciated.

We have recently identified two processes that may contribute to the natural antiadhesive mechanisms operating on the surface of fibrin clots. In particular, the adsorption of fibrinogen on various surfaces or pretreatment of fibrin clots with fibrinogen dramatically reduces adhesion of leukocytes and platelets [14,15]. This phenomenon has also been reported by others [13,16]. The mechanism by which fibrinogen renders hard surfaces non-adhesive is based on the formation of a ‘soft’ multilayered matrix characterized by weak adhesion forces and increased extensibility [17]. Accordingly, the origin of non-adhesive properties of fibrinogen matrices is their inability to transduce strong mechanical forces via integrin receptors, which results in insufficient signaling and weak cell spreading [15,17]. The second process depends on activation of plasminogen, which accumulates in a thin superficial layer of a fibrin clot, by the plasminogen activation system assembled on transiently adherent blood cells, including monocytes and platelets [18]. Consequently, the generated plasmin decomposes the superficial fibrin layer, resulting in cell detachment under flow. Although the antiadhesive effect of two fibrin-binding proteins, plasminogen and fibrinogen, involves proteolytic and non-proteolytic mechanisms, respectively, they are based on a common underlying mechanism: the alteration of a fibrin clot surface resulting in the formation of a mechanically unstable substrate that does not allow firm integrin-mediated cell attachment.

As a fibrin shell on the surface of thrombi in circulation is exposed to both fibrinogen and plasminogen, we have examined the combined effect of these proteins on cell adhesion,

anticipating a result that would be additive. Surprisingly, fibrinogen markedly decreased the antiadhesive effect of plasminogen. Using U937 monocytic cells, a cell line commonly used for studying the assembly of the plasminogen activating system [19–21], we have shown that fibrinogen interferes with activation of fibrin-bound plasminogen by cells.

Materials and Methods

Materials

Human plasminogen (depleted of plasmin activity), plasmin, thrombin and fibrinogen (depleted of fibronectin and plasminogen) were obtained from Enzyme Research Laboratories (South Bend, IN, USA). tPA was purchased from Calbiochem (Darmstadt, Germany) and uPA was obtained from Calbiochem (San Diego, CA, USA). Plasminogen and fibrinogen were labeled with ^{125}I using Pierce Iodination Beads (Thermo Scientific, Rockford, IL, USA), dialyzed against phosphate-buffered saline (PBS) and stored at $-20\text{ }^{\circ}\text{C}$. Fibrinogen and plasminogen concentrations were determined based on their extinction coefficients, 1.56 and $1.7\text{ at }1\text{ mg mL}^{-1}$, respectively. Fibrinogen conjugated with Alexa Fluor[®] 488 and the reagents for labeling plasminogen with the Alexa Fluor[®] 647 dye were purchased from Invitrogen (Carlsbad, CA, USA). Bovine serum albumin, phenylalanyl-L-prolyl-L-arginine chlormethyl ketone (PPACK) acid and polyvinylpyrrolidone (PVP) were from Sigma (St Louis, MO, USA). The chromogenic plasmin substrate D-Val-L-Leu-L-Lys *p*-nitroanilide hydrochloride (S-2251) was from Chromogenix Diapharma Group (Franklin, OH, USA). Calcein AM was purchased from Molecular Probes (Eugene, OR, USA).

Preparation of fibrin gels

Fibrin gels were formed in siliconized wells of 96-well format strips or 12-well format plates by mixing 60 μL or 250 μL , respectively, of 1.5 mg mL^{-1} fibrinogen in Hank's balanced salt solution (HBSS) with 0.25 U mL^{-1} thrombin for 2 h at $22\text{ }^{\circ}\text{C}$. Thrombin was inactivated by incubation with $50\text{ }\mu\text{M}$ PPACK and the gels were extensively washed with PBS. In selected experiments, the fibrin gels were prepared from ^{125}I -labeled fibrinogen to incorporate a total of $1.5\text{--}2.0 \times 10^5$ cpm per gel.

Cell adhesion assays

U937 monocytic cells were obtained from ATCC and cultured in RPMI 1640 supplemented with 10% fetal bovine serum. The cells were labeled with $5\text{ }\mu\text{M}$ calcein for 30 min, washed twice with HBSS + 0.1% BSA and assays were performed as described previously [14]. Briefly, aliquots (100 μL) of labeled U937 cells (5×10^5 per mL) in HBSS + 0.1% BSA were added to wells of a 96-well format plate and after 30 min incubation at $37\text{ }^{\circ}\text{C}$, the non-adherent cells were removed by two or three washes with PBS. Fluorescence was measured in a CytoFluorII fluorescence plate reader (Applied Biosystems, Foster City, CA, USA). For inhibition experiments, cells were mixed with different concentrations of fibrinogen before they were added to the wells. Alternatively, fibrin gels were incubated with various concentrations of soluble fibrinogen or plasminogen for 15 min at $22\text{ }^{\circ}\text{C}$, after which fibrinogen solutions were aspirated, gels rinsed and cells were added to the wells. Data are expressed as fluorescence of adherent cells.

Determination of plasminogen activation

Activation of plasminogen to plasmin was determined by either measuring fibrin degradation products or plasmin amidolytic activity using the chromogenic substrate S-2251. To measure fibrin degradation products, fibrin gels were prepared from ^{125}I -fibrinogen in 12-well format plates; 50–100 $\mu\text{g mL}^{-1}$ plasminogen (0.25 mL) was incubated with gels for 15 min at 22 °C. Gels were washed with PBS to remove unbound plasminogen, followed by the addition of U937 cells (6×10^5 per gel). After incubation for a specified period at 37 °C, the radioactivity of the supernatants containing fibrin degradation products was measured. To determine the plasmin amidolytic activity generated by cells adherent to immobilized plasminogen, the wells of 48-well Costar plates were coated with plasminogen (100 $\mu\text{g mL}^{-1}$), post-coated with 1%PVP and cell suspensions containing different numbers of cells were added for 20 min at 37 °C. Adherent cells were removed and the S-2251 color substrate was added (400 μL , 0.75 mM), followed by incubation for 2 h at 37 °C.

Confocal fluorescence microscopy

Fibrin gels were prepared by mixing 0.25 mL of 1.5 mg mL^{-1} fibrinogen in HBSS in glass/polycarbonate 50-mm Fluorodish™ dishes (World Precision Instruments, Sarasota, FL, USA) with 0.25 U mL^{-1} thrombin. After 2 h at 22 °C, thrombin was inactivated by PPACK and the gels were washed with PBS. The gels were then overlaid with 500 μL 1 mg mL^{-1} fibrinogen conjugated with Alexa Fluor 488 with or without plasminogen conjugated with Alexa Fluor 647 (100 $\mu\text{g mL}^{-1}$). Adsorption of fluorescently labeled proteins on the surface and in the bulk of fibrin gels was observed using a set-up based on a MicroTime200 Fluorescence lifetime microscope (PicoQuant, Berlin, Germany) and Olympus IX-71 inverted microscope (Center Valley, PA, USA) with 40× objective, which was previously described [22]. HEK 293 cells expressing green fluorescence protein (GFP) were used to identify the top surface of fibrin gels. Light intensity data were used for analyses.

Results

Antiadhesive effect of fibrinogen and plasminogen is not additive

We previously demonstrated that adsorption of either fibrinogen or plasminogen on the surface of fibrin gels produces a strong inhibition of leukocyte and platelet adhesion [14,18]. As shown in Fig. 1A, 1 mg mL^{-1} fibrinogen and 0.1 mg mL^{-1} plasminogen inhibited adhesion of U937 cells by ~ 70% and 90%, respectively. To investigate the possibility that the simultaneous addition of these proteins can produce a synergistic effect, fibrin clots were treated with fibrinogen and plasminogen at lower concentrations to produce ~ 50% inhibition, and then adhesion of U937 cells was examined. No additivity of inhibition was detected (Fig. 1B). Moreover, a different effect was observed when fibrinogen, plasminogen or both proteins were added to U937 cells that have already been adherent to the naked fibrin gels. While fibrinogen did not affect cell adhesion, apparently because of its inability to break the established integrin-fibrin bonds, plasminogen continued to exert an antiadhesive effect, indicating that generated plasmin was capable of digesting fibrin, resulting in the destabilization of the cell-fibrin attachment sites (Fig. 1C). However, when the same concentrations of fibrinogen and plasminogen were added together, the inhibitory effect of plasminogen was reduced compared with that of plasminogen alone (Fig. 1C). The

latter result suggested that the potential targets for fibrinogen may be the process of plasminogen activation or generated plasmin itself. In subsequent experiments, we examined the two possibilities.

Fibrinogen interferes with activation of plasminogen by U937 cells

To determine if fibrinogen can intervene with activation of fibrin-bound plasminogen, U937 cells were mixed with different concentrations of fibrinogen and then added to fibrin gels pretreated with plasminogen. As shown in Fig. 2A, fibrinogen blocked the formation of fibrin degradation products by generated plasmin. The effect was dose-dependent and at 2 mg mL⁻¹, fibrinogen produced ~ 85% inhibition of plasmin activity as determined by the measurement of ¹²⁵I-labeled fibrin degradation products released above the gels. Under the conditions of the present study, release of fibrin degradation products in the absence of plasminogen was negligible, suggesting that plasmin-mediated fibrinolysis by monocytic cells was superior to their non-plasmin dependent effects. Furthermore, BSA that was present during incubation of cells with fibrin gels at the concentration which on a molar basis exceeds that of fibrinogen did not influence cell adhesion to fibrin or generation of fibrin degradation products. It has been reported that fibrinogen can inhibit plasmin amidolytic activity directly [23]. To investigate the possibility that plasmin generated from fibrin-bound plasminogen was a target of fibrinogen, we examined the effect of fibrinogen on plasmin that was directly adsorbed on fibrin gels. Fibrin gels were incubated with plasmin (10 U mL⁻¹) for 1 min at 4 °C and the gels were extensively rinsed to remove non-bound plasmin. Solutions containing 1.5 mg mL⁻¹ fibrinogen were added above the gels and the radioactivity of solubilized fibrin was measured. Soluble fibrinogen did not influence the amount of fibrin degradation products released from the gels (Fig. 2B). The lack of direct influence of fibrinogen on plasmin suggests that fibrinogen interferes with the conversion of plasminogen into plasmin by cells.

It is known that fibrin-bound tPA stimulates activation of plasminogen [24,25] and is thought to be the primary plasminogen activator in the vasculature. Furthermore, direct uPA activation of fibrin-bound plasminogen may contribute to clot lysis [26,27]. Therefore, we examined the effect of fibrinogen on activation of fibrin-bound plasminogen by tPA and uPA (in the absence of cells). Plasminogen was adsorbed on the surface of fibrin gels, the gels were incubated with 10 ng mL⁻¹ tPA or 10 U mL⁻¹ uPA with and without fibrinogen for 2 h at 37 °C, and the solubilized radioactivity, as a measure of plasmin activity, was determined. At 1.5 mg mL⁻¹, the concentration that inhibited the generation of plasmin activity by cells by > 80%, fibrinogen did not significantly affect lysis of fibrin gels by plasmin generated by both plasminogen activators (Fig. 3). Together, the data indicate that fibrinogen affects neither plasmin immobilized on the surface of fibrin gels nor activation of fibrin-bound plasminogen by tPA and uPA, but rather interferes with the process of activation of fibrinbound plasminogen by cells and, hence, exerts its effect at the cell-fibrin interface.

Fibrinogen accumulates in a thin superficial layer of a fibrin gel and blocks activation of plasminogen by cells

We previously demonstrated that adsorption of fibrinogen on hard surfaces, including plastic, mica and glass, results in the formation of a non-adhesive multilayer matrix [15,17]. Moreover, pretreatment of fibrin clots with soluble fibrinogen leads to a dramatic reduction in cell adhesion [14,15], suggesting that fibrin may induce aggregation of fibrinogen. However, the accumulation of fibrinogen in a superficial layer of a clot has not been previously shown. To investigate the events occurring on the surface of fibrin clots, we initially examined the kinetics of adsorption of fibrinogen using a fluorescein-labeled tracer. Fibrin gels were incubated for various times with 1 mg mL⁻¹ fibrinogen containing tracer Alexa 488-labeled fibrinogen, and then binding was stopped by the addition of PBS followed by several washes. As shown in Fig. 4, fibrinogen bound in a time-dependent manner and significant amounts of the protein were associated with fibrin clots after 30 min. The examination of the kinetics of binding of fibrinogen labeled with ¹²⁵Iodine, a more traditional label, produced a similar isotherm of adsorption (Fig. 4). Coinciding with the deposition of fibrinogen was a rapid loss of adhesion of U937 cells (Fig. 4, inset).

To investigate the spatial distribution of fibrinogen in fibrin gels, the process of adsorption was monitored by confocal fluorescence microscopy. Fibrinogen (1 mg mL⁻¹) was present above the gels during the entire course of the experiment (30 min). Figure 5A represents three consecutive images of fibrin clots after the addition of tracer Alexa 488-fibrinogen. The deposition of fibrinogen on the top of the gels was observed as early as 2.5 min and then continued to increase. Quantification of the spatial distribution of the label showed that the concentration of fibrinogen above and inside the gels reached the maximal level after 30 min of incubation (Fig. 5B). However, the distribution of fibrinogen was not uniform, with a 2-fold higher concentration being present in a thin upper layer (~ 10 µm fluorescent band) than in the rest of the gel. After the removal of weakly bound fibrinogen by washing of fibrin clots for 5 and 60 min, fluorescence intensity on the top of the gels was ~ 15-fold higher than inside the gel, indicating that a significant portion of the protein remained associated with the superficial layer of a clot (Fig. 5C).

Plasminogen binds and concentrates in the superficial layer of fibrin gels [28,29] and may interfere with the occupancy by fibrinogen. To examine this possibility, fibrinogen (1 mg mL⁻¹) containing tracer Alexa 488-fibrinogen and plasminogen (0.1 mg mL⁻¹) containing tracer Alexa 647-plasminogen were added to fibrin gels simultaneously and their accumulation on the surface was detected Fig. 6C. Both proteins accumulated in the superficial layer and their presence was detected 1 h after dissociation of the label. After desorption for 60 min, both proteins were still present in the upper layer with the fluorescent intensity being ~ 18 and 3-fold higher on the top of the gel for fibrinogen and plasminogen, respectively, than inside the gels (Fig. 6B). Thus, the binding of plasminogen appears to be largely independent of fibrinogen deposition.

To demonstrate that the surface-bound fibrinogen was responsible for the inability of cells to activate plasminogen, fibrin gels were incubated with plasminogen and fibrinogen, followed by the removal of the proteins and the addition of U937 cells. As shown in Fig. 6C, cells adherent to the gels precoated with increasing concentrations of fibrinogen gradually lost

their ability to activate plasminogen, as measured by the release of ^{125}I -labelled fibrin degradation products. The concentration of fibrinogen required to inhibit activation of plasminogen by 50% was $\sim 0.45 \text{ mg mL}^{-1}$ and 90% inhibition was attained at a physiological concentration of 2 mg mL^{-1} . In contrast to the experimental conditions shown in Fig. 2, cells were added to fibrinogen that has been prebound to fibrin with no soluble fibrinogen present above the gels. Fibrinogen can potentially dissociate from the surface and when cells are added a new equilibrium will be established between free and surface-bound fibrinogen. However, only small amounts of fibrinogen ($\sim 12 \mu\text{g mL}^{-1}$; 35 nM) were found to dissociate from the surface of clots, which is insufficient to block monocyte receptors that bind fibrinogen with a K_d in a μM range [19]. Thus, the inability of cells to activate plasminogen can be attributed to fibrinogen associated with the surface of fibrin gels.

Fibrinogen masks the access of cells to immobilized plasminogen

The requirement for the cells to activate fibrin-bound plasminogen is evidently that they contact it. To begin to explore the mechanisms by which fibrinogen inhibits activation of plasminogen at the fibrin-cell interface, experiments with immobilized plasminogen were performed. For technical reasons, fibrin gels coated with plasminogen could not be used because as soon as cells engage and activate fibrin-bound plasminogen, they detach from the digested fibrin fibers. Therefore, plasminogen was immobilized onto plastic and the effect of fibrinogen was examined. In accordance with previous studies [30], immobilized plasminogen was capable of supporting strong integrin-mediated cell adhesion (Fig. 7A). As shown in Fig. 7B, adherent cells activated plasminogen; however, generated plasmin did not cause cell detachment (Fig. 7A). Furthermore, PPACK did not change the adhesion, confirming that both active and inactivated plasmin immobilized on plastic support adhesion [30]. This is opposite to fibrin-bound plasminogen where its activation by cells results in cell detachment. It is well established that plasminogen interacts with fibrinogen [31,32]. When fibrinogen was added to the immobilized plasminogen, this resulted in a marked decrease of cell adhesion (Fig. 7A) and reduced plasminogen activation (Fig. 7C). These results suggest that binding of fibrinogen to immobilized plasminogen masks it, preventing access to the plasminogen activators assembled on cells.

Discussion

In this study, we examined the interplay between two antiadhesive systems, non-proteolytic fibrinogen-dependent and proteolytic plasminogen-dependent, operating at the surface of fibrin clots. The major finding of the study is that when fibrin clots are exposed to fibrinogen and plasminogen simultaneously, as would occur in circulation, the deposition of fibrinogen on the surface of a clot prevents activation of fibrin-bound plasminogen by transiently adherent cells, indicating that the fibrinogen-dependent system counteracts the effect of the plasminogen-dependent antiadhesive system.

In previous studies, we demonstrated that the antiadhesive effect of fibrinogen adsorbed on various hard surfaces such as plastic, mica and glass arises from the formation of a multilayered matrix incapable of transducing strong mechanical forces via integrins, resulting in a weak cell adhesion [15,17]. Using atomic force microscopy (AFM) and AFM-

based force spectroscopy, we further showed that the deposition of fibrinogen in the form of a molecular bilayer is sufficient to render the surfaces poorly adhesive [17]. The treatment of fibrin clots with fibrinogen also results in the reduced adhesion of platelets and leukocytes [14,15]; however, it remained uncertain whether fibrinogen can accumulate on the surface of a clot. As the characterization of fibrinogen adsorption on fibrin clots is not readily amenable to AFM, we investigated this process using confocal fluorescence microscopy. Here, we show for the first time that fibrinogen accumulates in a thin superficial layer of a clot, where its concentration remains several-fold higher than in the bulk of the gel even after extensive washing (Fig. 5). Plasminogen, which is also known to accumulate in the superficial layer of a clot [29], does not interfere with fibrinogen deposition (Fig. 6). The intrinsic ability of fibrinogen to aggregate upon contact with various surfaces implies that it may accumulate in the upper layer of fibrin clots in the form of a non-adhesive multilayer.

When deposited on the surface of fibrin clots alone, plasminogen exerts a potent antiadhesive effect resulting from its activation to plasmin by adherent cells and nicking of fibers at the cell-fibrin contact sites [18]. The precise mechanism by which the simultaneous adsorption of fibrinogen and plasminogen abolishes activation of plasminogen by cells will require further study. However, the simplest interpretation of the observed phenomenon, which is also supported by our data shown in Fig. 7, is that the generated fibrinogen matrix masks the surface-bound plasminogen and precludes its physical contact with cells. Indeed, the overlay of immobilized plasminogen with fibrinogen blocked activation of plasminogen, apparently by creating a barrier between plasminogen and adherent cells. It is likely that the high concentration of fibrinogen used in our experiments to duplicate a ~ 10:1 ratio of fibrinogen to plasminogen in circulation was a major factor in its dominating effect. Alternatively, the deposited fibrinogen matrix can induce rearrangement in the interaction of integrin $\alpha_M\beta_2$ (Mac-1), a major fibrinogen receptor on monocytes, with uPAR, the receptor for uPA. Previous studies have demonstrated that $\alpha_M\beta_2$ forms a functional unit with uPAR [33,34], and both receptors cooperate during adhesion of activated blood monocytes to fibrinogen [35]. Determining the mechanisms by which the multilayer fibrinogen may influence the signaling and/or proteolytic functions of the $\alpha_M\beta_2$ /uPAR-uPA system will be an important direction for future investigation.

Our previous finding that fibrin-bound plasminogen can be activated by transiently adherent U937 cells [18] is in agreement with studies that used THP-1 monocytic cells [36]. Indeed, without fibrinogen, an assay system that has been used in both of these studies, U937 and THP-1 cells activate fibrin-bound plasminogen efficiently. Based on this finding, Dejouvencel *et al.* [36] have proposed that the conversion of fibrin-bound plasminogen by uPA-bearing cells (i.e. through a process involving the contact of two surfaces) represents a new mechanism of plasmin formation. The finding of the present study that fibrinogen can repress activation of fibrin-bound plasminogen brings the universality of the proposed model into question and suggests that the relationship between fibrin and monocytes may differ in various locations. Thus, the activation of fibrin-bound plasminogen at the interface between fibrin clots and adherent cells may not occur in circulation where fibrinogen is expected to be deposited on the clot surface and to interfere with plasminogen activation. On the other hand, uPA-bearing monocytes that migrate inside the clots through the vessel wall [10] may

directly activate plasminogen prebound to fibrin fibers because the clot's interior is depleted of soluble fibrinogen and not exposed to blood flow.

It is important to note that activation of fibrin-bound plasminogen can be induced not only by U937 monocytic cells but also by isolated peripheral blood monocytes [18]. However, in contrast to U937 cells that secrete uPA constitutively and also display uPAR-bound uPA, freshly isolated blood monocytes do not contain detectable levels of uPA on their surface [20]. Furthermore, because functional plasma levels of tPA under normal physiological conditions are very low (20 pM) and blood monocytes bind tPA with a K_d in the μM range [19], this apparently results in the loss of tPA during monocyte isolation even though tPA was initially bound to cells. The levels of both activators are substantially elevated only during blood clotting and inflammation. Therefore, isolated blood monocytes need to be supplemented with either exogenous uPA or tPA to activate fibrin-bound plasminogen. Thus, it appears that both U937 cells and blood monocytes have limitations as experimental models. Further work will be necessary to define the effect of fibrinogen on activation of fibrin-bound plasminogen by other blood cells, including platelets, as well as its effect *in vivo*.

The existence of two potent antiadhesive mechanisms, fibrinogen- and plasminogen-dependent raises an issue of their potential contribution to the non-adhesive properties of the fibrin clot surface. Because of the clear superiority of the fibrinogen-dependent system, it is uncertain under which circumstances the plasminogen-dependent system may play a significant role. One of the possibilities is the heterogeneity of the clot surface. Recent studies using a novel technique that allowed the imaging of the thrombi developed in large vessels demonstrated that the surface of clots displayed variable spatial intensities of the platelet-, fibrin- and fibrinogen-specific fluorophores, indicating a non-uniform coverage of clots with fibrinogen [7]. Therefore, plasminogen and fibrinogen may potentially accumulate at different regions of the clot surface. Another possibility is that the contribution of the two antiadhesive systems may be altered in pathological conditions. Further studies of the distribution of fibrinogen and plasminogen on the surface of clots *in vivo* and in model systems *in vitro* may help to define the localization and roles of the two antiadhesive systems.

The surface-mediated control of thrombus growth and stability is a new concept. Although the fact that the surfaces of stable hemostatic thrombi are covered with fibrin has been long known, the paradox of why fibrin, a highly adhesive substrate for platelets and leukocytes *in vitro*, does not effectively support adhesion of these cells in circulation has not been addressed. We have previously proposed [18] that the reason for the loss of reactivity of fibrin clots for platelets and leukocytes is the ability of fibrin to bind both fibrinogen and plasminogen, resulting in the assembly of a potent antiadhesive system that guards clots from excessive cell accumulation. This protective mechanism may fulfill a dual purpose. First, it will prevent the accumulation of platelets, thereby halting the overall process of thrombus propagation. Second, it will prevent the recruitment of leukocytes whose premature adhesion with a concurrent release of proteolytic enzymes may compromise the thrombus stability essential for early hemostasis. The efficiency of both fibrinogen- and plasminogen-dependent processes observed *in vitro* in conjunction with supporting evidence

from *in vivo* studies suggests their physiological relevance. Thus, surface-mediated control of thrombus growth and stability may represent a previously unrecognized aspect of hemostasis. The finding of the present study that the fibrinogen-dependent system counteracts the antiadhesive effect of the plasminogen-dependent system points to additional complexity in the relationship between the two processes that warrants future functional analyses of their differential contribution to normal hemostasis.

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Fig. 1.

Effect of fibrinogen and plasminogen on adhesion of U937 cells to fibrin clots. (A) Fibrin gels prepared as described in Materials and Methods were preincubated with solutions (100 μ L) of 1 mg mL⁻¹ fibrinogen (Fg) or 0.1 mg mL⁻¹ plasminogen (Pg) for 30 min at 22 °C. Solutions above the gels were aspirated, the gels were briefly washed with PBS and aliquots of calcein-labeled U937 cells were added. Adhesion was determined as described in Materials and Methods. Adhesion of control U937 cells to untreated fibrin gels was assigned a value of 100% (denoted C). (B) Fibrin gels were preincubated with solutions of fibrinogen (50 μ g mL⁻¹), plasminogen (50 μ g mL⁻¹) or both for 30 min at 22 °C. The liquid above the gels was aspirated, the gels were washed with PBS and adhesion of U937 cells was determined. (C) U937 cells were allowed to adhere to untreated fibrin gels for 30 min at 37 °C, non-adherent cells were removed and 100 μ L-aliquots of fibrinogen (1 mg mL⁻¹), plasminogen (50 μ g mL⁻¹) or both were added to the gels with adherent cells. After incubation for another 30 min at 37 °C, cell adhesion was determined by measuring fluorescence of adherent cells. Data are means $\pm$ SD of quadruplicate determinations from three to four experiments.

Fig. 2.

Soluble fibrinogen inhibits activation of fibrin-bound plasminogen by U937 cells. (A) Fibrin gels were prepared by mixing 1.5 mg mL^{-1} fibrinogen ($250 \text{ }\mu\text{L}$ per well) and thrombin in the presence of tracer ^{125}I -fibrinogen. Thrombin was inactivated with PPACK and non-bound radioactivity was extracted by prolonged washing of the gels with PBS. The total radioactivity of the gels was $174000 \pm 3200 \text{ cpm}$. The gels were incubated with $100 \text{ }\mu\text{g mL}^{-1}$ plasminogen ($250 \text{ }\mu\text{L}$) for 10 min at $22 \text{ }^{\circ}\text{C}$, and then washed until no plasminogen was found in the extracts as detected by its conversion into plasmin in the presence of tPA (10 ng mL^{-1})

and measuring plasmin activity using S-2251 (0.75 mM). Suspensions of U937 cells in HBSS (6×10^5 in 300 μ L HBSS + 0.1% BSA) containing different concentrations of fibrinogen were added to the gels for 30 min at 37 °C and then radioactivity released above the gels was measured. Radioactivity dissociated from the gels without added plasminogen (220 ± 130 cpm) was subtracted. (B) Fibrin gels were preincubated with plasmin (10 U mL⁻¹) for 1 min at 4 °C, the gels were extensively rinsed to remove non-bound plasmin and then the aliquots containing 1.5 mg mL⁻¹ fibrinogen (●) or only buffer (○) were added. The gels were incubated at 37 °C and solubilized radioactivity was measured at indicated times. Radioactivity released from the gels without added plasmin is also shown (▲). Data are means of triplicate determinations, and error bars represent standard errors. The results shown are representative of three independent experiments.

Fig. 3.

Soluble fibrinogen does not influence activation of fibrin-bound plasminogen by tPA and uPA. Fibrin gels were prepared by mixing 1.5 mg mL^{-1} fibrinogen ($250 \text{ } \mu\text{L}$ per well) and thrombin in the presence of tracer ^{125}I -fibrinogen. The gels were extensively washed to remove fibrinogen that was not incorporated into the gels, incubated with plasminogen ($50 \text{ } \mu\text{g mL}^{-1}$) for 15 min at $22 \text{ } ^\circ\text{C}$ and non-bound plasminogen was washed out. Aliquots ($250 \text{ } \mu\text{L}$) containing tPA (20 ng mL^{-1}) or uPA (10 U mL^{-1}) with (grey bars) or without (open bars) 1.5 mg mL^{-1} fibrinogen were added to the gels and solubilized radioactive products were measured after 2 h at $37 \text{ } ^\circ\text{C}$. Controls included gels without adsorbed plasminogen and gels with adsorbed fibrinogen only. Data are means of triplicate determinations, and error bars represent standard errors. The results shown are representative of two independent experiments.

Fig. 4.

The kinetics of fibrinogen binding to fibrin gels. Fibrin gels formed in 96-well format strips were incubated with 1 mg mL^{-1} solutions of fibrinogen ($100 \mu\text{L}$) containing Alexa 488-labeled fibrinogen tracer ($\circ$) or ^{125}I -fibrinogen ($\bullet$) for various times. The binding was stopped by the addition of PBS ($200 \mu\text{L}$), the gels were washed to remove non-bound fibrinogen and then radioactivity or fluorescence was measured. The data shown are the average of four measurements at each experimental data point and are representative of two to three experiments. Inset: adhesion of U937 cells to fibrin gels incubated with 1 mg mL^{-1} fibrinogen was determined as described above.

Fig. 5.

Fibrinogen accumulates in a thin superficial layer of fibrin clots. (A) Fibrin gels prepared in 50-mm Fluorodish™ glass dishes were incubated with 1 mgmL^{-1} fibrinogen containing tracer Alexa 488-labeled fibrinogen. Confocal microscopy images of the ZX plane of a gel were made 2.5, 5 and 10 min after the addition of fibrinogen. The $\alpha_M\beta_2$ -expressing HEK 293 cells transfected with GFP were used to identify the fibrin gel's surface (arrowhead). A vertical bar on the right shows the position of the glass surface, the bulk of fibrin gel, the gel's surface and solution above the gel. A color scale bar on the left represents the relative

intensity in photons per pixel, with the lowest intensity corresponding to the glass surface. (B) Cross-sections of the gels incubated with Alexa 488 fibrinogen for 2.5, 5, 10 and 30 min averaged by 64 consecutive pixels. (C) Images of a representative gel after 2 h incubation with tracer Alexa 488-fibrinogen (1 mg mL^{-1}) and washing with PBS for 5 and 60 min at 22°C . The table on the right shows the distribution of fluorescence intensity in the solution above the gel, in the superficial layer of a gel, inside the gel and inside the glass in photons/pixel. The intensity data shown are the averaged areas.

Fig. 6.

Fibrinogen deposited on the surface of fibrin clot prevents activation of fibrin-bound plasminogen by U937 cells. (A) Fibrin gels were incubated with tracer Alexa 488-fibrinogen (1 mg mL^{-1}) and tracer Alexa 647-plasminogen ($100 \mu\text{g mL}^{-1}$) for 30 min at 22°C and images were made 1 h after washing of weakly bound proteins with PBS. (B) Cross-sections of the gels incubated with fluorescently labeled fibrinogen and plasminogen averaged by 64 consecutive pixels. (C) Plasminogen (0.1 mg mL^{-1}) was adsorbed on the surface of fibrin gels prepared from ^{125}I -labeled fibrinogen ($182\,000 \pm 6100 \text{ cpm per gel}$) as described in

Fig. 3 and then aliquots (250 μ L) containing various concentrations of fibrinogen were added to the gels for 15 min at 22 °C. Fibrinogen solutions were aspirated, gels were washed with 1 ml PBS and U937 cells (6×10^5) were added for 60 min at 37 °C. To estimate the amount of plasmin generated by adherent cells, gels were covered with 500 μ L PBS and 125 I-fibrin degradation products released in the solution after 60 min incubation were measured. The result shown is representative of three independent experiments.

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Fig. 7.

Adhesion of U937 cells to immobilized plasminogen and its activation by adherent cells. (A) The wells of 96-well microtiter plates were coated with $10 \mu\text{g mL}^{-1}$ plasminogen overnight at 4°C , blocked with 1% polyvinylpyrrolidone (PVP) followed by incubation with $100 \mu\text{L}$ PBS (control) or $100 \mu\text{g mL}^{-1}$ fibrinogen for 20 min at 22°C . Solutions were aspirated and adhesion of calcein-labeled U937 cells (5×10^4 per $100 \mu\text{L}$) was measured in the presence or absence of PPACK ($100 \mu\text{M}$). (B) The wells of 48-well Costar plates were coated with plasminogen ($100 \mu\text{g mL}^{-1}$), post-coated with 1% PVP and cell suspensions ($100 \mu\text{L}$) containing different numbers of cells were added for 20 min at 37°C . Adherent cells were removed and the S-2251 color substrate was added ($400 \mu\text{L}$, 0.75 mM) followed by incubation for 2 h at 37°C . (C) Fibrinogen ($100 \mu\text{L}$, $100 \mu\text{g mL}^{-1}$) was added to the wells coated with $100 \mu\text{g mL}^{-1}$ plasminogen and incubated at 22°C for 20 min. Solutions were aspirated and the plate was placed on ice to minimize activation of plasminogen. Cells (5×10^5 per $400 \mu\text{L}$) were added, the plate was centrifuged at $500 \times g$ for 2 min and then incubated for 20 min at 37°C . Adherent cells were removed and generated plasmin activity was measured with S-2251. Activity of immobilized plasminogen without added fibrinogen served as a control.