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ORIGINAL ARTICLE

Blood clot contraction differentially modulates internal and external fibrinolysis

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Essentials

- Clot contraction influences the rate of fibrinolysis *in vitro*.
- Internal fibrinolysis is enhanced ~2-fold in contracted vs. uncontracted blood clots.
- External fibrinolysis is ~4-fold slower in contracted vs. uncontracted blood clots.
- Contraction can modulate lytic resistance and potentially the clinical outcome of thrombosis.

Summary. *Background:* Fibrinolysis involves dissolution of polymeric fibrin networks that is required to restore blood flow through vessels obstructed by thrombi. The efficiency of lysis depends in part on the susceptibility of fibrin to enzymatic digestion, which is governed by the structure and spatial organization of fibrin fibers. How platelet-driven clot contraction affects the efficacy of fibrinolysis has received relatively little study. *Objective:* Here, we examined the effects of clot contraction on the rate of internal fibrinolysis emanating from within the clot to simulate (patho)physiological conditions and external fibrinolysis initiated from the clot exterior to simulate therapeutic thrombolysis. *Methods:* Clot contraction was prevented by inhibiting platelet myosin IIa activity, actin polymerization or platelet-fibrin(ogen) binding. Internal fibrinolysis was measured by optical tracking of clot size. External fibrinolysis was determined by the release of radioactive fibrin degradation products. *Results and Conclusions:* Clot contraction enhanced the rate of internal fibrinolysis ~2-fold. In contrast, external fibrinolysis was ~4-fold slower in contracted clots. This dichotomy in the susceptibility of contracted and uncontracted clots to internal vs. external lysis suggests that the rate of lysis is dependent upon the interplay between accessibility of fibrin fibers to fibrinolytic agents, including clot permeability, and the spatial proximity of the fibrin fibers that modulate the effects of the fibrinolytic enzymes. Understanding how compaction of blood clots influences clot lysis might have important implications for prevention and treatment of thrombotic disorders.

Keywords: blood clotting; clot retraction; fibrin; fibrinolysis; platelets.

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Introduction

Blood clots are typically dissolved by the fibrinolytic system after fulfillment of their hemostatic functions [1–3]. Clot dissolution results from proteolytic cleavage of the fibrin network, a scaffold formed during coagulation through thrombin-catalyzed cleavage of fibrinogen into polymerizing fibrin monomers. Fibrin is cleaved by plasmin, which is converted from the circulating zymogen plasminogen into an active protease by tissue-type plasminogen activator (t-PA) or urokinase plasminogen activator (u-PA). Fibrin provides a matrix that binds plasminogen and t-PA, thereby enhancing the rate of plasmin generation approximately 500-fold [4,5]. Plasmin also converts single chain t-PA and u-PA into their more active two-chain forms. In addition, plasmin cleaves peptide bonds in fibrin, exposing carboxyl-terminal lysine residues that provide additional binding sites for t-PA and plasminogen [6]. Thus, the initial breakdown of fibrin leads to exposure of additional binding sites for t-PA and plasminogen,

which furthers plasmin generation and generates a positive feedback loop [7].

Fibrinolytic activity in blood is downregulated in part by thrombin-activatable fibrinolysis inhibitor, plasminogen activator inhibitors and α2-antiplasmin. Lysis is also strongly influenced by intrinsic properties involving fibrin structure and composition [8,9], including variations in fiber diameter, orientation and network porosity [8,10–13]. For example, individual thin fibers are cleaved at a faster rate than thicker fibers. However, dense fibrin networks composed of many thin fibers take longer to lyse than a network composed of thick fibers [9,14,15]. Lysis of individual fibers proceeds uniformly across the fibrin fiber, which is dependent on crawling of plasmin molecules along the fiber diameter [9], a process that is amplified by exposure of C-terminal lysines [16]. Lysis is also modulated by the inherent strain that develops during formation of the fibrin network, which augments the lysis of individual fibrin fibers [8]. However, the application of strain to a preformed fibrin clot impedes lysis [8,17,18]. The application of strain may also affect plasminogen binding and/or the propensity of fibers to elongate [8]. Crosslinking of fibrin by factor XIIIa also reduces lysis by incorporating α2-antiplasmin, reduces the binding affinity of plasmin to fibrin, and alters the mechanical properties of the clot, which affects fiber extensibility and stiffness [18–20]. Fibrin structure also influences the permeability of the network, which in turn affects transport of t-PA, plasminogen and plasmin through the clot [21].

Lysis is also influenced by the incorporation of cells in the clot architecture. Contraction, also referred to as retraction, is the volume shrinkage of blood clots [22] and results in the expulsion of serum [23,24]. Activated platelets generate contractile forces and, because of platelet–fibrin interactions, these forces are propagated through the fibrin network [25]. Contraction also changes the structure of the fibrin network and reduces clot permeability [1,26]. This leads to formation of dense fibrin networks that are resistant to fibrinolysis [27–29]. Incorporation of erythrocytes also influences fibrinolysis by leading to the formation of thicker fibers with fewer branch points [10,30] and by reducing permeability [31,32], which has been attributed to compression of erythrocytes and formation of tessellated arrays of polyhedral-like cells [31]. The expulsion of serum can also change the content of pro- and antifibrinolytic agents within the contracting clot [32]. Clot contraction is impaired in a variety of (pro)thrombotic conditions, such as ischemic stroke, deep vein thrombosis, systemic lupus erythematosus and asthma [33–39]. However, the effect of clot contraction on lysis has not been subject to detailed study.

Fibrinolysis occurs physiologically when clot formation and fibrinolysis are triggered concurrently or sequentially (internal fibrinolysis) [15] or by administration of lytic agents such as recombinant t-PA in the clinical setting of thrombosis (external fibrinolysis) [40]. It is often assumed that the same factors regulate clot lysis through both pathways, but possible dichotomies in the regulation of internal and external fibrinolysis have not been explored in detail. Here, we examine how clot contraction of whole blood clots affects internal and external fibrinolysis. Our data show that contraction doubles the rate of internal fibrinolysis but markedly impairs external fibrinolysis. These differences suggest that the rate of lysis is determined by the interplay between accessibility of fibrin to fibrinolytic agents, including clot permeability, and spatial proximity of the fibrin fibers that modulate the effects of the fibrinolytic enzymes.

Methods

Sample preparation

Blood was collected from healthy individuals following informed consent in accordance with the guidelines established by the Institutional Review Board at the University of Pennsylvania or the Ethical Committee of the Interregional Clinical Diagnostic Center (Kazan, Russian Federation) and the Declaration of Helsinki. Blood was drawn into 3.8% trisodium citrate 9:1 by volume, stored at room temperature and used within 4 h.

Initiation and inhibition of clot contraction

Blood clotting and clot contraction were initiated by addition of 1 U mL^{-1} thrombin and 2 mM $CaCl_2$ to whole blood collected in citrate (0.32% final concentrations), conditions shown previously to be optimal for rapid clot formation, prevention of RBC settling and robust clot compaction that was almost complete within 30–60 min [41]. Following activation, samples were rapidly transferred to plastic 12 mm × 7 mm × 1 mm cuvettes that were precoated with a residual layer of 4% Triton X-100 and the cuvettes were inserted into a thermostatic chamber of the Thrombodynamics Analyzer System at 37 °C. The change in clot size was tracked based on measuring the optical properties of the clot every 15 s for 20 min (Video S1). The extent of clot contraction was determined by comparing clot size relative to its size at $t = 0$. To compare fibrinolysis in contracted and uncontracted blood clots, contraction was prevented with the following inhibitors: abciximab (ReoPro®, Eli Lilly; final concentration 100 μg mL^{-1}) to block interaction of fibrin (ogen) with the αIIbβ3 integrin receptor on platelets; latrunculin A (Abcam; final concentration 4 μM) to disrupt actin polymerization; or blebbistatin (Sigma-Aldrich; final concentration 200 μM) to inhibit myosin IIa. Although these inhibitors also affect other platelet functions, the fact that each inhibitor caused a comparable marked reduction in clot contraction (Fig. 1) suggests that their other effects are secondary.

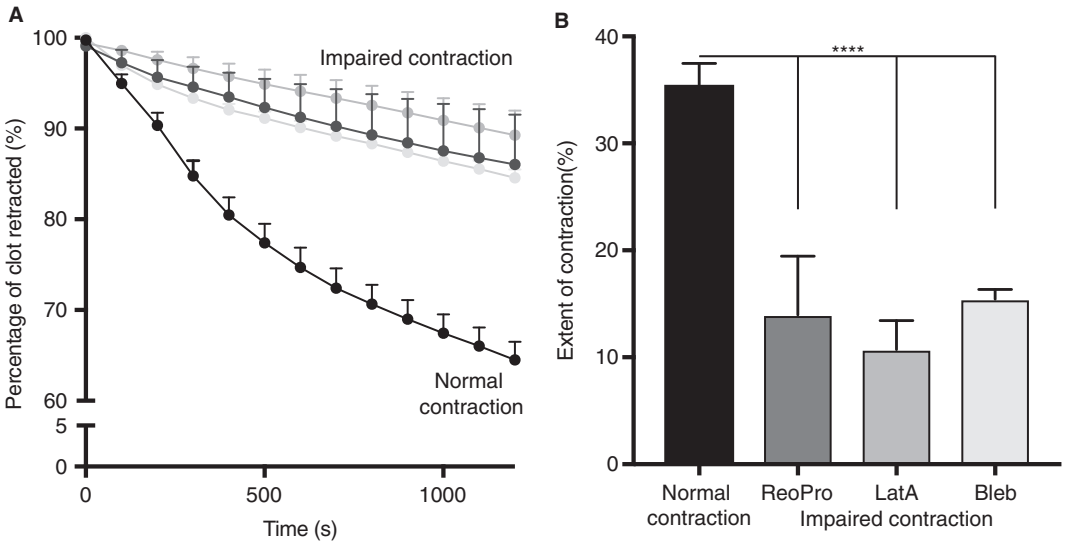

Fig. 1. Inhibition of clot contraction. ReoPro® (abciximab), latrunculin A (LatA) and blebbistatin (Bleb) were used to impair (A) the kinetics and (B) final extent of clot contraction. Statistical analysis was completed using a one-way ANOVA, ****$P < 0.0001$. $n > 3$. Data represented as mean ± SEM.

Internal fibrinolysis

Internal fibrinolysis was measured using the Thrombodynamics Analyzer (HemaCore, Moscow, Russian Federation) optical tracking system, which is designed to quantify changes in clot growth [42] or shrinkage [41] based on the light scattering properties through the use of a red-light-emitting diode and a CCD camera. t-PA (final concentration 75 ng mL^{-1}) was added to citrated whole blood; clotting and contraction were then initiated by adding 1 U mL^{-1} thrombin and 2 mM $CaCl_2$ (final concentrations). We optimized the t-PA concentration such that lysis did not begin until after clot formation had been completed, as determined using dynamic turbidimetry (Figure S4). Following activation with thrombin, samples were rapidly transferred to plastic 12 mm × 7 mm × 1 mm cuvettes that were precoated with a residual layer of 4% Triton X-100 and the cuvettes were inserted into a thermostatic chamber of the Thrombodynamics Analyzer System at 37 °C. The change in clot size was tracked optically every 100 s over 3.5 h (Video S1). A control blood sample from the same donor without t-PA added was measured in parallel to distinguish between clot diminution as a result of fibrinolysis and that caused by contraction. Time to resolution or complete lysis was determined as a 95% reduction in relative clot size, as 5% is within the optical limitations of the system.

External fibrinolysis

To measure external fibrinolysis, citrated whole blood was mixed with trace amounts of ^{125}I-labeled fibrinogen (PerkinElmer, Waltham, Massachusetts). Clotting was again initiated by adding 1 U mL^{-1} thrombin (Sigma-Aldrich, St. Louis, Missouri) and 2 mM $CaCl_2$ (final concentrations). Clots were transferred to borosilicate tubes pre-lubricated with 4% Triton X-100 to prevent fibrin from sticking. Clots were allowed to form and contract for 30 min following addition of thrombin and then overlaid with 200 μL of phosphate-buffered saline (PBS) containing t-PA (75 ng mL^{-1} final concentration). Samples were incubated at 37 °C and the release of radioactive soluble fibrin cleavage products was measured in 10-μL samples that were collected from the surrounding buffer at 0 and 30 min, 1, 2, 3, 4 and 24 h using a gamma-counter (PerkinElmer Life and Analytical Sciences, Waltham, Massachusetts). Data were analyzed as the number of counts per minute in the collected buffer relative to the total amount of radiolabeled fibrinogen in the clot. The amount of released radioactive soluble fibrin cleavage products was normalized to the amount of radioactivity present in the surrounding buffer at the time of t-PA addition. Clots were formed from the blood of three independent donors with samples made in triplicate from each donor.

To examine if the serum expelled from contracting blood clots (which may contain pro- and antifibrinolytics) affects the rate of lysis, the ^{125}I-containing clots were prepared as described, but after contraction had been allowed to proceed for 30 min, the expelled serum was removed and replaced with PBS or PBS containing plasminogen or platelet-poor plasma obtained from the same blood sample. Clots were incubated at 37 °C for an additional 15 min, overlaid with 200 μL of PBS containing tPA (75 ng mL^{-1} final concentration), and the radioactivity in the supernatant was measured over time.

Fitting and statistical analyses

Fibrinolysis kinetic curves were fit using a linear fit from 0 to 60 min for external lysis and an exponential decay for

internal lysis for 0–4 h. For internal lysis the rate of fibrinolysis (k) was calculated using an exponential decay curve where individual experimental curves were fit and a rate constant (k) was solved for and averaged. The fitting equation was: relative clot size = (initial clot size − plateau)− k*time + plateau. Time to 50% lysis was determined by ln (2)/k. All statistics were completed using Prism GraphPad 7.0 (GraphPad Software, San Diego, CA, USA). Samples were analyzed for significance using a two-way ANOVA with an $\alpha = 0.05$ between samples with normal and impaired contraction.

Results

Contracted clots remain susceptible to fibrinolysis

Clot contraction was induced by the addition of thrombin to whole blood containing radiolabeled fibrinogen. In the absence of exogenous t-PA, the release of radiolabeled degradation products was only increased significantly at 24 h (Figure S1), indicating low levels of internal fibrinolysis catalyzed by endogenous fibrinolytic enzymes. When clot contraction was initiated and followed 30 min later by addition of t-PA (75 ng mL^{-1}), degradation products were released by 2 h (Figure S1). When t-PA was added at the same time that clot formation was initiated, lysis was evident by 1 h, as assessed by optical tracking (Movie S1). These results show that blood clots maintain their accessibility to fibrinolytic enzymes and susceptibility to lysis, notwithstanding the mechanical stresses, reduced permeability and structural rearrangements induced by contraction. However, it is not clear from these experiments if clot contraction affects the rate of fibrinolysis.

Effect of clot contraction on external fibrinolysis

To address this question, we first investigated how clot contraction affects external fibrinolysis induced by t-PA applied to preformed clots that had undergone partial or more extensive contraction. Aliquots of whole blood were allowed to clot and contract for 30 min in the absence or presence of inhibitors (ReoPro, latrunculin A or blebbistatin) prior to adding t-PA, and the release of soluble radioactive fibrin degradation products was measured over time. Clots with impaired contraction released 2–4-fold more degradation products during the first 30 min and continued to lyse at a rate 4-fold faster than contracted clots over the initial 4 h following addition of t-PA (Fig. 2).

The same difference in the rates of lysis was seen when the serum expelled from contracted clots was replaced with phosphate-buffered saline or platelet-poor plasma (Figure S2). Neither the removal of serum nor supplementation with plasminogen affected the kinetics of fibrinolysis after addition of t-PA. These results indicate that inhibitors of fibrinolysis potentially present in serum expelled from contracted clots were not responsible for the reduction in the rate of external lysis and that sufficient endogenous plasminogen had been incorporated into contracted blood clots to allow fibrinolysis to occur. Therefore, the observed differences in lysis can be attributed to the effect of contraction on fibrin.

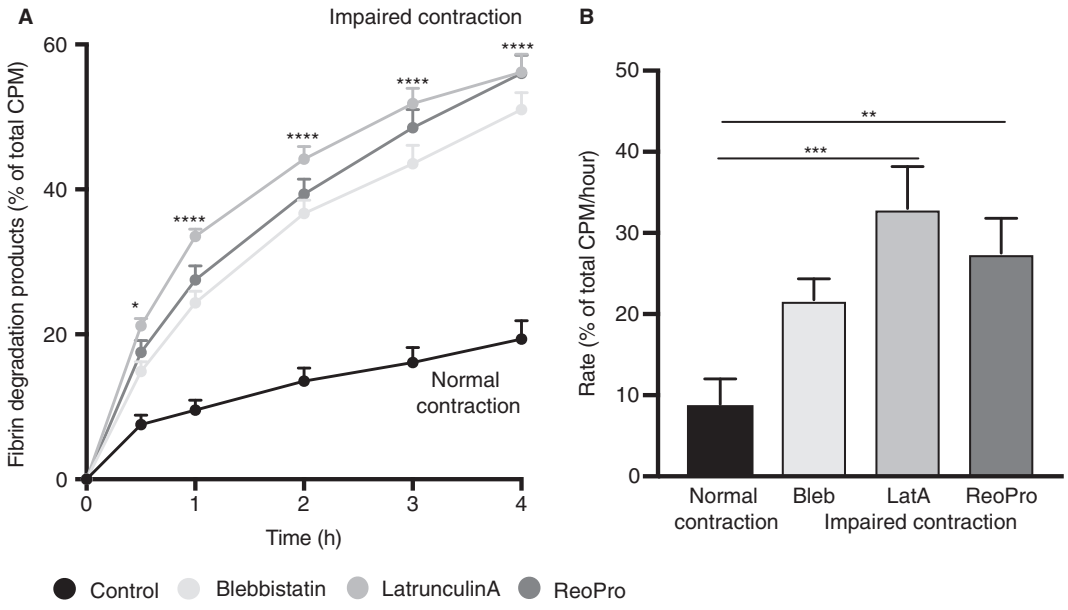

Fig. 2. Effect of clot contraction on the rate of external fibrinolysis. t-PA (75 ng mL^{-1}) was added 30 min after formation of blood clots in the absence or presence of inhibitors of clot contraction ReoPro® (abciximab), latrunculin A and blebbistatin for the (A) kinetics and (B) rate. The release of soluble radioactive fibrin degradation products, measured as count per minute (CPM), over time was used as a measure of fibrinolysis. Data are represented as mean ± SEM. Statistical analysis was performed using a one-way ANOVA where $*P < 0.05$, $****P < 0.0001$. $n > 3$.

Effect of clot contraction on internal fibrinolysis

To assess the effects of clot contraction on internal fibrinolysis, we added t-PA to citrated samples of whole blood prior to adding thrombin, thus permitting t-PA be incorporated throughout the clot volume. The presence of t-PA significantly reduced clot size beyond the effect of contraction alone and this difference became evident at ~25 min after initiation of blood clotting and platelet activation with thrombin (Figure S3, Movie S1B,C). This time was taken thereafter as the starting point of fibrinolysis. Contracted clots underwent complete lysis, as determined by a loss of optical density below 5% of the initial level, at a rate that was 4–4.5 fold faster ($P < 0.001$) than with clots formed in the presence of inhibitors of contraction (Fig. 3, Table 1). Accordingly, the average time to 50% lysis increased about 3–4-fold and the average time to complete lysis was 1.7–2 fold longer for clots with contraction impaired by blebbistatin, latrunculin A, and ReoPro compared to contracted clots ($P < 0.0001$).

Discussion

Clot formation and lysis are accompanied by dramatic changes in fibrin architecture, mechanical tension on individual fibers, cellular composition and permeability, among other alterations. Clots also undergo contraction, a process of remodeling that both stabilizes the fibrin seal and fosters blood flow around potentially obstructive thrombi. However, the effects of the contraction process on clot lysis have not been studied in detail. This study provides a direct comparison of how clot contraction affects internal and external fibrinolysis.

Strong, albeit indirect, evidence that clot contraction occurs intravitally in blood vessels comes in part from structural studies. It was recently shown that contraction of blood clots *in vitro* results in the deformation of red blood cells to polyhedral shapes or polyhedrocytes. [31] Based on this observation, the presence of polyhedrocytes can be considered as an objective indication that clot contraction has occurred and therefore can be used to investigate the intravital contraction of clots and thrombi analyzed *ex vivo*. With this in mind, compressed polyhedrocytes, a morphological sign of clot contraction, have been found in thrombi extracted from coronary arteries of patients with ST-elevation myocardial infarction [43], venous thrombi and hemostatic clots [36,44], and pulmonary emboli [37]. These findings support the concept and reality of intravascular intravital clot contraction.

The pathogenic significance for thrombosis of deficits of clot contraction has been emphasized by studies showing that clot contraction is diminished in patients with ischemic stroke [33], venous thromboembolism [34] and systemic lupus erythrematosis [35], leading to more obstructive thrombi. On the other hand, the importance of clot contraction *in vivo* is also exemplified by the discovery of disorders that disrupt the generation and propagation of contractile force and thus predispose individuals to clot instability and bleeding. Development of

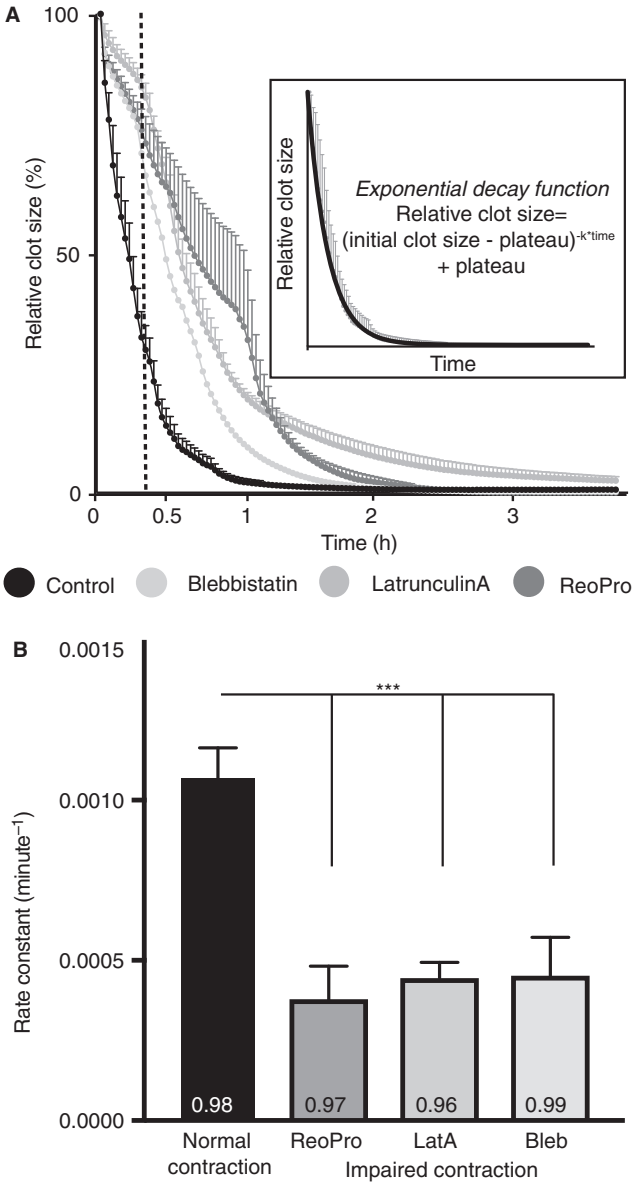

Fig. 3. Effect of clot contraction on the rate of internal fibrinolysis. t-PA (75 ng mL^{-1}) was added to the blood before clotting was initiated by thrombin and $CaCl_2$ in the absence or presence of inhibitors of clot contraction ReoPro® (abciximab), latrunculin A and blebbistatin. (A) The reduction in clot size was measured to assess fibrinolysis or, in the case of the control, the combined effects of contraction and fibrinolysis. The 25-min time-point is marked with a vertical dashed black line to indicate when fibrinolysis begins. Insert shows a representative curve fit (black) of a contracted clot (gray). (B) The rate of lysis was calculated using fitting analysis with an exponential decay function. The fitting parameters corresponding to the experimental conditions applied are shown in Table 1. Statistical analysis was completed using a one-way ANOVA. ***$P < 0.0001$. $n = 3$. Data represented as mean ± SEM. The average goodness of fit data for each experimental condition, r^2 values, are listed on the respective bars.

Table 1 Kinetic parameters of the t-PA-induced internal lysis of contracted blood clots and clots with contraction impaired by the inhibitors

	Rate of lysis, k ($s^{-1} \times 10^{-3}$)	Time to 50% lysis (s)	Time to complete lysis (s)
Contracted clots	1.7 ± 0.7	596 ± 80	2660 ± 380
Contraction prevented with blebbistatin	0.45 ± 0.09***	1720 ± 450*	4520 ± 410*
Contraction prevented with latrunculin A	0.44 ± 0.03***	2020 ± 440**	5830 ± 380**
Contraction prevented with ReoPro	0.38 ± 0.08***	1570 ± 110*	8830 ± 1300****

The parameters were determined from a fitting analysis using an exponential decay (see Methods for details). Statistical analysis of uncontracted compared to contracted clots was completed using a one-way ANOVA, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Time to complete lysis corresponds to the time to reach 95% reduction in relative clot size.

transgenic diYF mice, which allows for the selective impairment of outside-in signaling, has revealed that tyrosine phosphorylation of β3 within the ICY motif is essential for clot contraction. Importantly, these diYF mice, which have normal platelet numbers, size and aggregation, but impaired contraction, have a bleeding phenotype [45,46]. Deletion of Bcl-3 in mouse platelets impairs, whereas overexpression of Bcl-3 enhances, clot contraction and clot stability [47]. Mutation or loss of non-muscle myosin IIa in humans or mice results in decreased clot contraction, decreased thrombus stability and an increased tendency to bleed, although the significance of these results is ambiguous because these conditions are accompanied by disorders of platelet production and macrothrombocytopenia [48–52]. Collectively, these data provide evidence of a role for platelet-mediated contraction in clot stability and hemostasis and in thrombosis.

Because contraction of clots and thrombi occurs *in vivo*, it is highly likely to modulate endogenous fibrinolysis and responsiveness to thrombolytic agents. The compaction of clots would be expected to impair infiltration by fibrinolytic enzymes, thereby contributing to the well-established relationship between clot maturity (i.e. extent of contraction) and resistance to fibrinolysis. Indeed, a direct relationship between clot contraction and endogenous fibrinolysis has been recently described *in vivo* [53]. These and other evidence-based considerations motivated us to study the effects of clot contraction on fibrinolysis, which has potential physiologic and pathophysiological implications.

However, the determinants of internal and external fibrinolysis may differ. We studied whole blood clots that contain a large fraction of erythrocytes, which is primarily a reasonable model for venous clots or thrombi. To understand how clot contraction affects fibrinolysis, we used two complementary model systems, one simulating internal lysis of intravascular clots and the other simulating therapeutic (external) lysis with thrombolytic agents. Internal and external fibrinolysis were each assessed using two methodologies, optical tracking and quantifying cleavage products, but there were technical challenges that precluded using them in parallel. Measuring the release of radiolabeled fibrin for internal fibrinolysis was limited because of difficulties distinguishing between lysed fibrin and background radioactivity. The use of optical tracking in internal lysis allowed us to separate contraction and lysis. In contrast, optical tracking was not well suited for measuring external fibrinolysis (i.e. addition of t-PA after clot contraction has occurred). Because the thickness of the reaction chamber is relatively small compared to its width and length, the movement of the lytic agent in the serum surrounding and within contracted clots and those with impaired contraction might differ. This difference in area exposed to t-PA might thereby dominate the rate of lysis. Although there is a difference in surface of the clots for the release of radiolabeled fibrinogen, this influence was smaller than the differences seen in lysis, because the entire clot could be immersed in the t-PA-containing solution. Although it is likely that slight differences arise as a result of the methodology, these distinctions do not influence the qualitatively differential response of contracted vs. uncontracted clots to internally or externally applied t-PA.

Our data show that contraction impedes external fibrinolysis, consistent with previous studies that compared outcomes of t-PA added to platelet-rich vs. platelet-poor plasma [26] and clots formed in the presence of increasing numbers of platelets [54]. In other previously described models, clots were sectioned prior to lysis, which may alter surface area and structural rearrangements that occur as a result of clot contraction [55], or fibrinolysis was assessed in the presence of ultrasound, which promotes transport of t-PA [56,57]. Therefore, we used a model in which the composition of the clot is maintained and the impact of contraction *per se* could be delineated more clearly. In addition, our experiments examined the effects of contraction on lysis of whole blood clots containing RBCs, a model previously explored using other methods [58].

There are several plausible mechanistic explanations for the impairment of external lysis (Fig. 4): (i) the clot is less permeable to t-PA and plasminogen and, perhaps less likely, to fibrin degradation products extruded during the contraction process; (ii) reduced surface area accessible to t-PA; (iii) formation of dense unstructured fibrin–platelet aggregates [59]; (iv) increased fibrin network density [15]; (v) increased mechanical tension on individual fibrin fibers leading to greater stability [17,60]; (vi) t-PA binding

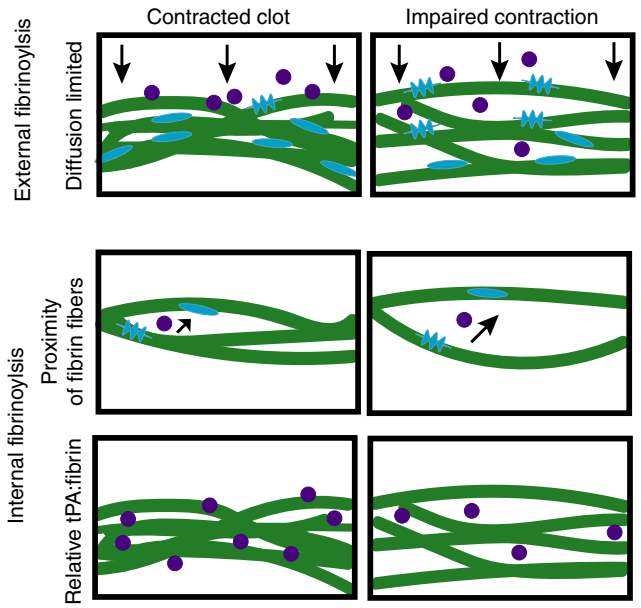

Fig. 4. Proposed mechanism underlying the effect of clot contraction on fibrinolysis. Fibrin is represented in green, plasmin in blue and t-PA in purple. We propose that external fibrinolysis is influenced primarily by clot permeability impaired by contraction, whereas internal fibrinolysis is more dependent on the proximity of fibrin fibers to each other and reduction in clot volume induced by contraction, both of which increase the t-PA to fibrin ratio. Note that additional mechanisms are described in the Discussion.

to fibrin outcompeting plasmin binding [61]; (vii) activation within the contracted clot of antifibrinolytic enzymes such as carboxypeptidases-B and -N, which cleave off C-terminal lysines from fibrin and thus limit the binding sites for t-PA and plasminogen, preventing plasmin formation and fibrinolysis [62,63]; and (viii) depletion of t-PA near the clot surface because of the lack of flow [64].

There is evidence to support the contribution of each of these mechanisms. Clot permeability is clearly reduced during contraction, as platelets and fibrin compact RBCs within the fibrin core [31], which impairs the rate at which t-PA moves through the clot volume. Contraction also reduces clot volume and thereby the surface area upon which fibrinolysis occurs. It is also likely that contraction alters the structure of the fibrin network itself. Less porous fibrin clots lyse more slowly than clots composed of loose fibers, which has been attributed to a reduction in fibers per volume [9,32,65,66]. Taken together, our findings may help to explain how older clots that are more likely to have undergone intravital contraction become less amenable to exogenous thrombolytics [67].

We asked whether the primary reason external lysis is impaired during the contraction process is that inhibitors (e.g. plasminogen activator inhibitor-1 secreted from activated platelets) are expelled from the clot. However, no differences were observed in the rate of fibrinolysis of contracted clots immersed into extruded serum, phosphate-buffered saline, plasminogen or platelet-poor plasma. These results show that in our system fibrinolysis occurred primarily as a result of plasminogen that had been incorporated into the clot during its formation. These results emphasize the importance of the physical changes in fibrin itself that develop during contraction in the development of resistance to external lysis.

The outcomes were remarkably different when the effect of contraction on internal lysis was modeled by adding t-PA prior to initiating clotting in order to recapitulate the physiological sequence of reactions [68]. The concentration of t-PA added (75 ng mL^{-1}) did not initiate lysis until after the clot had been completely formed, as confirmed by a kinetic turbidimetry assay (Figure S4). At a higher t-PA concentration, lysis could occur before the clot structure was established and contraction began. At a lower t-PA concentration, fibrinolysis may be delayed or incomplete. It is noteworthy that although circulating t-PA concentrations have been reported at levels of ~5 ng mL^{-1} [69], secondary hyperfibrinolysis in response to intravascular fibrin deposition leads to a burst in the concentration of t-PA in the blood of up to 50–95 ng mL^{-1} [70,71]. In therapeutic thrombolysis, the circulating level of recombinant t-PA is very high initially but decreases soon after injection to levels of around 50 ng mL^{-1} [72]. Therefore, the 75-ng mL^{-1} concentration of t-PA used in this study may or may not correspond roughly to the actual concentration of circulating t-PA in either natural hyperfibrinolysis or soon after initiation of therapeutic thrombolysis. Importantly, this concentration of t-PA does not inhibit platelet function [73]. However, with all the variables of the conditions for *in vivo* lysis, including flow, size and location of clot or thrombus, and local concentrations of all proteins, no one knows the most relevant details for realistic modeling, so more experimentation is needed to more fully appreciate the role of clot contraction.

The rate of internal fibrinolysis did not depend on clot permeability because t-PA and plasminogen already reside within the clot [9]. Clot contraction enhanced internal fibrinolysis, consistent with previous studies (although none of them involved analysis of clots formed with whole blood) [74], probably because the redistribution [31] enables individual plasmin molecules to move between fibers more efficiently [15,66]. In support of this concept, it has been observed through changes in optical density that lysis appears to occur along a front that stretches across the surface of the clot. The reduction in clot volume that occurs as a result of contraction will also increase the local concentration of t-PA and plasminogen, accelerating plasmin generation. In contrast to external lysis, this more rapid generation of plasmin may contribute to accelerated lysis before carboxypeptidases-B and -N can act. An additional potential mechanism for differences observed between internal and external fibrinolysis involves the inhibitory effect of factor XIIIa on fibrinolysis [20], which has been attributed

to the incorporation of α2-antiplasmin into the clot [75–77]. The inhibitory effect of factor XIIIa-mediated crosslinking is more profound in clots that have undergone compaction [20]. In addition, the incorporation of other inhibitors of fibrinolysis, such as plasminogen activator inhibitor 1 and 2, von Willebrand factor, binding to GP1b, and the actions of carboxypeptidases-B, -U and -N, may contribute to the observed differences in fibrinolysis [78].

The *in vitro* assays employed in this study were designed to simulate key aspects of natural internal and therapeutic external thrombolysis, permit direct comparisons between these two important clinical scenarios and create experimental settings in which the effects of clot contraction itself could be assessed. Because much is yet unknown about the actual conditions for fibrinolysis *in vivo*, our experiments are as yet only a relatively crude approximation of reality. Nevertheless, our studies provide new insights into how clot contraction may enhance internal fibrinolysis as well as reduce vascular obstruction, and they clarify the effects of clot aging on impaired response to therapeutic lysis. These findings have the potential to inform the development of more targeted and selective therapeutics with respect to the ability of hemostatic clots and obstructive thrombi to undergo intravital contraction. For example, our results suggest that by modulating clot contraction, it may be possible to make thrombi or clots more or less sensitive to natural or therapeutic thrombolysis. Identifying and targeting rate-limiting steps in clot contraction may enhance both physiologic understanding and management of pathologic thrombosis.

Addendum

R. I. Litinov, V. Tutwiler, D. B. Cines and J. W. Weisel designed the research; V. Tutwiler, A. D. Peshkova, G. L. Minh and S. Zaitsev performed experiments; V. Tutwiler, R. I. Litinov and J. W. Weisel analyzed data; V. Tutwiler, R. I. Litinov, D. B. Cines and J. W. Weisel wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interests.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Fig. S1. Spontaneous vs. t-PA-induced external fibrinolysis assessed by the release of radioactive soluble fibrin degradation products.
Fig. S2. Effect of the medium surrounding the clot on the rate of external fibrinolysis.
Fig. S3. Reduction in size of contracting clot in the absence of t-PA (contraction alone) verses in the presence of t-PA (contraction plus lysis).
Fig. S4. Turbidity curve for clot formation followed by internal fibrinolysis in the presence of 75 ng mL^{-1} t-PA added to blood plasma before clotting induced by 1 U mL^{-1} thrombin and 25 mM $CaCl_2$.
Movie S1. Tracking of clot contraction and internal fibrinolysis with optical assay.

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