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blood advances

Red blood cell aggregation within a blood clot causes platelet-independent clot shrinkage

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Key Points

- RBCs suspended in platelet-depleted plasma undergo aggregation within a thrombin-induced clot and cause platelet-independent clot shrinkage.
- RBC-induced clot shrinkage may reinforce blood clot contraction, especially in thrombocytopenia and/or impaired platelet contractility.

Platelet-driven blood clot contraction (retraction) is important for hemostasis and thrombosis. Red blood cells (RBCs) occupy approximately half of the clot volume, but their possible active contribution to contraction is unknown. The work was aimed at elucidating the ability of RBCs to promote clot shrinkage. To distinguish the effects of platelets and RBCs, we formed thrombin-induced clots from reconstituted human samples containing platelet-free plasma and platelet-depleted RBCs, followed by tracking the clot size. The clots before and after RBC-induced shrinkage were analyzed using histology and scanning electron microscopy. Tension developed in the RBC-containing plasma clots was measured with rheometry, and theoretical modeling was used to elucidate the clot shrinkage mechanisms. Platelet-depleted clots formed in the presence of RBCs exhibited >20% volume shrinkage within one hour. This process was insensitive to blebbistatin, latrunculin A, and abciximab. At a higher RBC count, clot shrinkage increased, whereas in the absence of RBCs no plasma clot shrinkage was observed. At low platelet counts, RBCs stimulated clot contraction proportionately to the platelet level. Inside the shrunken clots, RBCs formed aggregates. The average tensile force per 1 RBC was ~120 ± 100 pN. Clots from purified fibrinogen formed in the presence of RBCs did not change in size, but underwent shrinkage in the presence of osmotically active dextran. Blood clot shrinkage can be caused by RBCs alone, and this effect is because of the RBC aggregation driven mainly by osmotic depletion. The RBC-induced clot shrinkage may reinforce platelet-driven blood clot contraction and promote clot compaction when there are few and/or dysfunctional platelets.

Introduction

Contraction or retraction of blood clots is the process of clot shrinkage and structural remodeling[1,2] that occurs both in vitro and in vivo.[3] It affects the course and outcomes of thrombosis[3-8] and is also important in hemostasis.[3,9] Contraction of blood clots occurs by activated platelets attached to fibrin fibers.[10,11] Platelet contractility is driven by the interaction of nonmuscle myosin IIA with actin.[12,13] The intracellular force is transmitted to fibrin via the integrin αIIbβ3,[14] resulting in compaction of the clot.[1,11] During

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The full-text version of this article contains a data supplement.

contraction, a blood clot undergoes redistribution of components, such that the platelet-fibrin meshwork is accumulated at the clot periphery, whereas red blood cells (RBCs) are condensed within the clot core[1] and become tightly packed to a polyhedral shape, hence named "polyhedrocytes" or "piezocytes."[1,15,16]

The role of RBCs in clot contraction remains poorly understood.[17] The extent and rate of clot shrinkage are inversely related to the volume fraction of RBCs in blood (hematocrit).[18,19] At the same time, the deformation of RBCs activates the mechanosensitive membrane cationic channels Piezo1, followed by reduction of RBCs in size and surface exposure of phosphatidylserine, increasing the generation of endogenous thrombin,[20] and increased thrombin activity further augments platelet contractility. Notably, blood clot contraction depends not only on the quantity but also on the quality of RBCs, such that RBCs with increased rigidity reduce clot contraction compared with normal softer RBCs.[21,22]

Analysis of in vitro blood clots and in vivo thrombi has shown that often RBCs comprise the main constituent, sometimes reaching >80% of the clot volume fraction,[1,6,15,23-25] although after clot contraction the RBC packing is extremely tight, owing to platelet-induced compression.[15,26] It is unknown whether RBC aggregation can play a role in blood clot formation and structural rearrangements.

At low shear rates, RBCs form linear (rouleaux) or 3-dimensional (3D) aggregates,[27-29] which promotes venous thrombosis.[30,31] There are 2 mechanisms proposed for such RBC aggregation, namely the bridging and the depletion models. In the bridging model, plasma proteins (mainly fibrinogen) "bridge" the adjacent cells to form aggregates.[32-34] In the depletion model, aggregation occurs because the concentration of osmotically active macromolecules near RBC surfaces is depleted compared with their concentration in the bulk phase, resulting in an osmotic pressure gradient and a net "depletion" force.[35,36]

This study aimed to explore whether RBC aggregation can induce blood clot shrinkage per se, in the absence of platelets, and, if yes, what the mechanisms involved are. Our experiments have demonstrated that RBC aggregation plays a remarkable role in the process of blood clot shrinkage.

Material and methods

For detailed methods, see Section 1 of the supplemental Material.

Platelet-depleted blood clots and dynamics of RBC-induced clot shrinkage

The mixtures of washed RBCs and platelet-free plasma (PFP) from the same donor referred to as platelet-depleted reconstituted blood samples (supplemental Figures 1-3) were clotted to follow subsequent clot shrinkage.[18] Briefly, clot formation and further compaction either in the blood (natural or reconstituted) or platelet-rich plasma (PRP) samples were induced by adding 2 mM $CaCl_2$ and 1 U/mL human thrombin. The thrombin-activated blood or plasma samples were transferred to cuvettes prelubricated with a residual layer of a detergent to prevent fibrin sticking to the walls. The cuvette was placed into a chamber (37°C) of the thrombodynamics analyzer (HemaCore), and the clot size was tracked for 60 minutes. The serial clot images were converted computationally into kinetic curves (relative extent of clot shrinkage vs time) that were analyzed for the extent of clot contraction/shrinkage at the end point (60 minutes), area under the curve, average velocity of clot contraction/shrinkage, and the lag time (supplemental Figure 4).

Measurement of tension generated by platelet-depleted blood clots

Clots from reconstituted platelet-depleted blood samples were formed between horizontal rheometer plates, and the clot tension was measured continuously as a negative normal (vertical) pulling force.[37] The RBC-related tension was determined as the difference between the maximal normal forces generated by the clots in the presence and absence of RBCs.

Continuum theory

To better understand the mechanisms underlying the RBC-induced clot compaction, we minimize the free energy in a continuum model for fibrin clots containing RBC (for details, see Section 3 of the supplemental Material). The model builds on the continuum analysis of fibrin gels[38] by adding the contributions of aggregated RBC to the free energy. Briefly, the elastic energy density of the fibrin network is based on an 8-chain model of polymer elasticity as adapted to fibrin and described previously.[39,40] The energy density of the bridging interactions of RBCs is taken to be neo-Hookean given that it represents the elastic energy stored in stretched (or compressed) biopolymer chains anchored in the membranes of adjacent RBC.[41] The energy density of interactions between RBC and fibrin is based on measured adhesive interactions between RBC and fibrinogen.[42] The model also includes the energy density associated with electrostatic and depletion interactions of RBC based on the theory of Neu and Meiselman[43] and the free energy density of the mixing of liquid and other components of the clot based on a Flory-Rehner type model that is used in elastomeric gels.[38]

The study involving human subjects was approved by the University of Pennsylvania Institutional Review Board.

Results

Differential effects of platelet inhibitors on the contraction of clots formed in whole blood and PRP

While studying the effects of various platelet inhibitors on blood clot contraction, we discovered that their efficacy was different in whole blood and PRP. To suppress platelet contractility, we applied a number of inhibitors at highly effective concentrations (see the supplemental Methods): PGE_1 (increases cyclic adenosine monophosphate level), latrunculin A (an inhibitor of actin polymerization), blebbistatin (an inhibitor of nonmuscle myosin IIa), abciximab (a Fab-antibody fragment, inhibitor of fibrin interaction with the integrin αIIbβ3), atopaxar (PAR1 antagonist), and tirofiban (synthetic integrin αIIbβ3 inhibitor). Expectedly, the inhibitors suppressed all the parameters of clot contraction (Figure 1; supplemental Table 1). The only exception was the effect of PGE_1, which prolonged the lag time and reduced the area under the kinetic curve, but did not significantly reduce the average velocity and final extent of clot contraction. However, in whole blood, none of the inhibitors suppressed clot contraction completely, allowing for the residual 70% to 85% of the initial clot size (except for PGE_1). When the same platelet inhibitors at the same concentrations were added to PRP, the contraction of PRP clots was almost

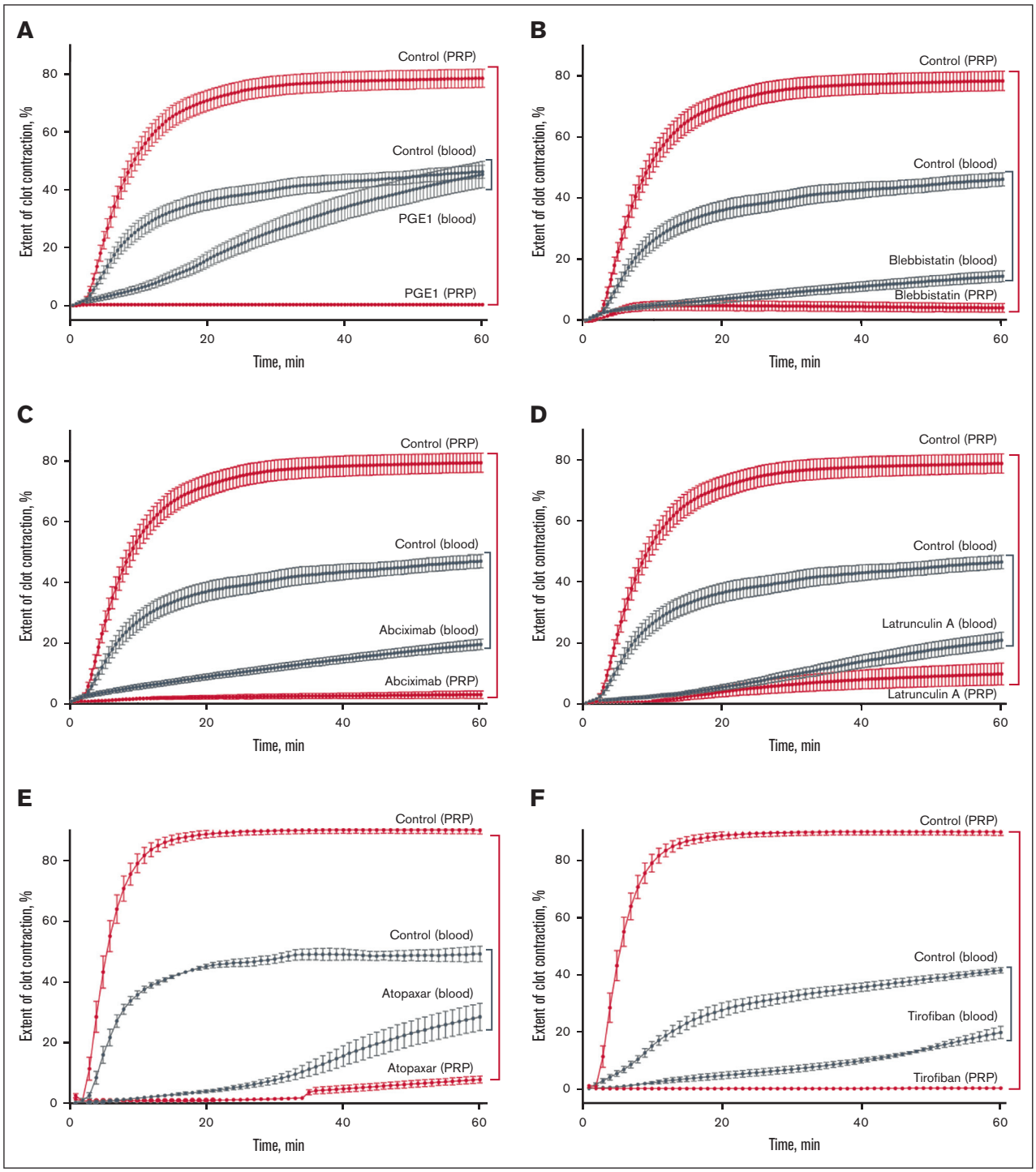

Figure 1. Comparative kinetic curves of platelet-driven clot contraction in whole blood (black) vs PRP (red) in the absence (control) and presence of the following platelet inhibitors. 1 μM PGE_1 (A), 200 μM blebbistatin (B), 100 μg/mL abciximab (C), 4 μM latrunculin A (D), 10 μM atopaxar (E), and 25 μM tirofiban (F). Note that the inhibitory effects on clot contraction in whole blood are only partial, whereas in PRP the inhibition is complete. The citrated blood or plasma samples were preincubated with the inhibitors at 37°C for 3 minutes before the initiation of clotting and clot contraction by thrombin (1 U/mL). The averaged curves from 4 or 5 experiments with blood samples from independent donors are presented as the mean ± standard error of the mean.

completely prevented compared with whole blood, that is, retaining almost 100% of the initial clot size after 1 hour (Figure 1; supplemental Table 1). In particular, the inhibitors completely abolished clot contraction in PRP from normal ~90% down to 0, whereas in whole blood the inhibitors reduced the extent of contraction from normal 45% to 51% down to 15% to 23%, that is,

twofold to threefold (with the exception of PGE_1) (supplemental Table 1). The inhibitory effect of ticagrelor on clot contraction was moderate, although ticagrelor was still more efficient in PRP than whole blood (supplemental Figure 5; supplemental Table 1).

These results suggest that blood clot compaction may be caused by RBCs and that this effect, unlike the platelet-driven clot compression, is unrelated to the intracellular contractile actomyosin machinery associated with αIIbβ3-mediated transmission of the traction force to the extracellular fibrin network.

To rule out the possibility that the difference in the inhibitors' efficacy between whole blood and PRP was because of absorbance by RBCs, requiring higher concentrations, we performed experiments on whole blood clot contraction at various concentrations of the inhibitors and plotted the dose-response data (supplemental Figure 6). The results show that at the final concentrations >100 μM for blebbistatin, >2 μM for latrunculin A, >50 μg/mL for abciximab, >5 μM for atopaxar, and >10 μM tirofiban, the final extent of contraction remained constant at a level of ~20% to 25%. Therefore, the results obtained confirm that the distinct difference in sensitivity to the inhibitors between whole blood and PRP is real and not because of a concentration shift.

The results obtained confirm the key role of platelet contractility, but they also suggest that there are noncontractile cellular reactions, likely related to the presence of RBCs, that can also contribute to the fibrin clot shrinkage.

Shrinkage of clots formed from reconstituted blood samples in the absence of platelets or in the presence of a low platelet count

To see whether clot compaction can be driven by forces other than platelet contractility, we induced clot formation in the platelet-depleted reconstituted blood samples prepared by mixing washed RBCs and PFP from the same donor at a physiological RBC count of 4×10^6/μL. Continuous registration of the clot size revealed a progressive volumetric shrinkage that reached >20% of the initial clot size within 1 hour after thrombin-induced clot formation (Figure 2A). Importantly, control clots formed in the same PFP without RBCs remained visually unchanged within the time of observation. The results indicate that fibrin clots in plasma can be partially compacted in the absence of platelets owing to the presence of RBCs. This process was insensitive to the inhibitors of nonmuscle cell contractility, such as blebbistatin, latrunculin A, and abciximab, given that none of the kinetic parameters of the RBC-induced clot shrinkage were affected (Figure 3; supplemental Table 2). In parallel control experiments, the inhibitors applied at the same concentrations effectively suppressed the platelet-driven clot contraction (Figure 1; supplemental Table 1). The results obtained clearly show that blood clot compaction may be caused by RBCs and that this effect, unlike the platelet-driven clot compression, is unrelated to the intracellular contractile actomyosin machinery associated with αIIbβ3-mediated transmission of the traction force to the extracellular fibrin network. Notably, the RBC-induced clot shrinkage increased with an increase of the RBC count from 4×10^6/μL to 6×10^6/μL, which caused a significant increment in the extent and average velocity of clot shrinkage without affecting the lag time and area under the kinetic curve (supplemental Figure 7; supplemental Table 3), confirming that the process is RBC dependent.

Although the complete absence of platelets in blood is hardly possible, there are a number of pathological conditions where the platelet-driven clot contraction is compromised and the RBC-induced clot compaction could be substantial. To test this presumption, we investigated the effects of RBCs on the extent of clot shrinkage in the presence of low platelet counts (20×10^3/μL,

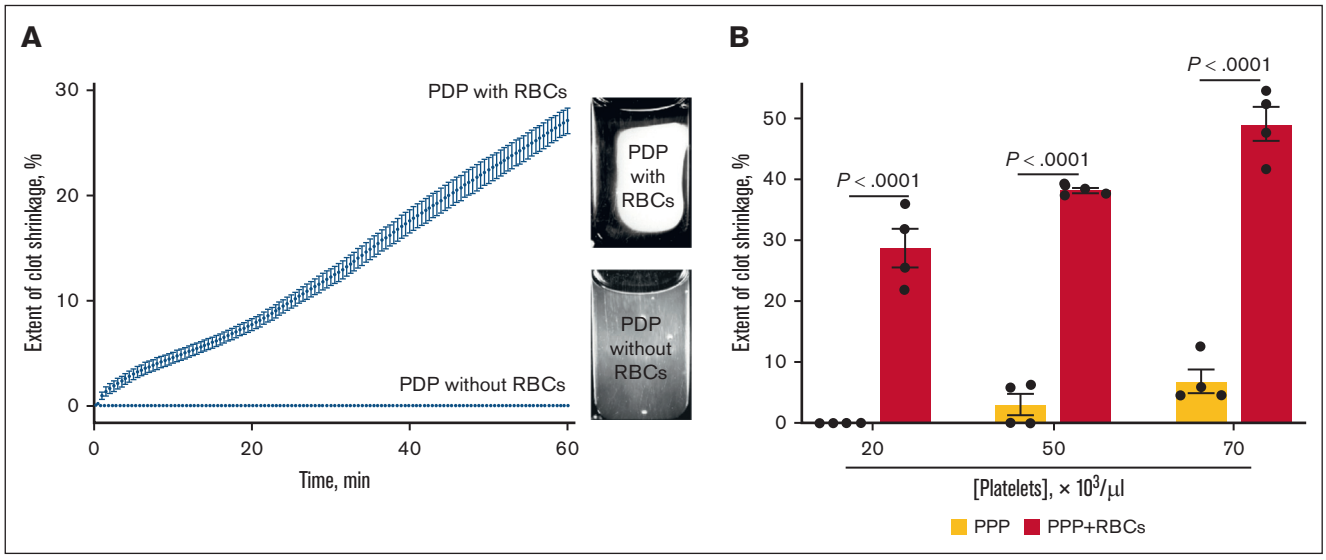

Figure 2. Role of RBCs in clot shrinkage under varying conditions. (A) Comparative kinetic curves and representative photographic images showing clots formed from PDP with RBCs (shrunken clot) or without RBCs (unshrunk clot). (B) At low platelet counts (mimicking thrombocytopenia), RBCs promote clot shrinkage proportionately to the platelet content. The final extent of clot shrinkage was measured in PPP containing 20×10^3/μL, 50×10^3/μL, and 70×10^3/μL either without or with addition of 4×10^6/μL RBCs. Results from 4 experiments with plasma and washed RBC of independent donors are presented as a mean ± standard error of the mean. Statistical significance of the observed differences was confirmed using a paired 2-tailed *t* test. PDP, platelet-depleted plasma; PPP, platelet-poor plasma.

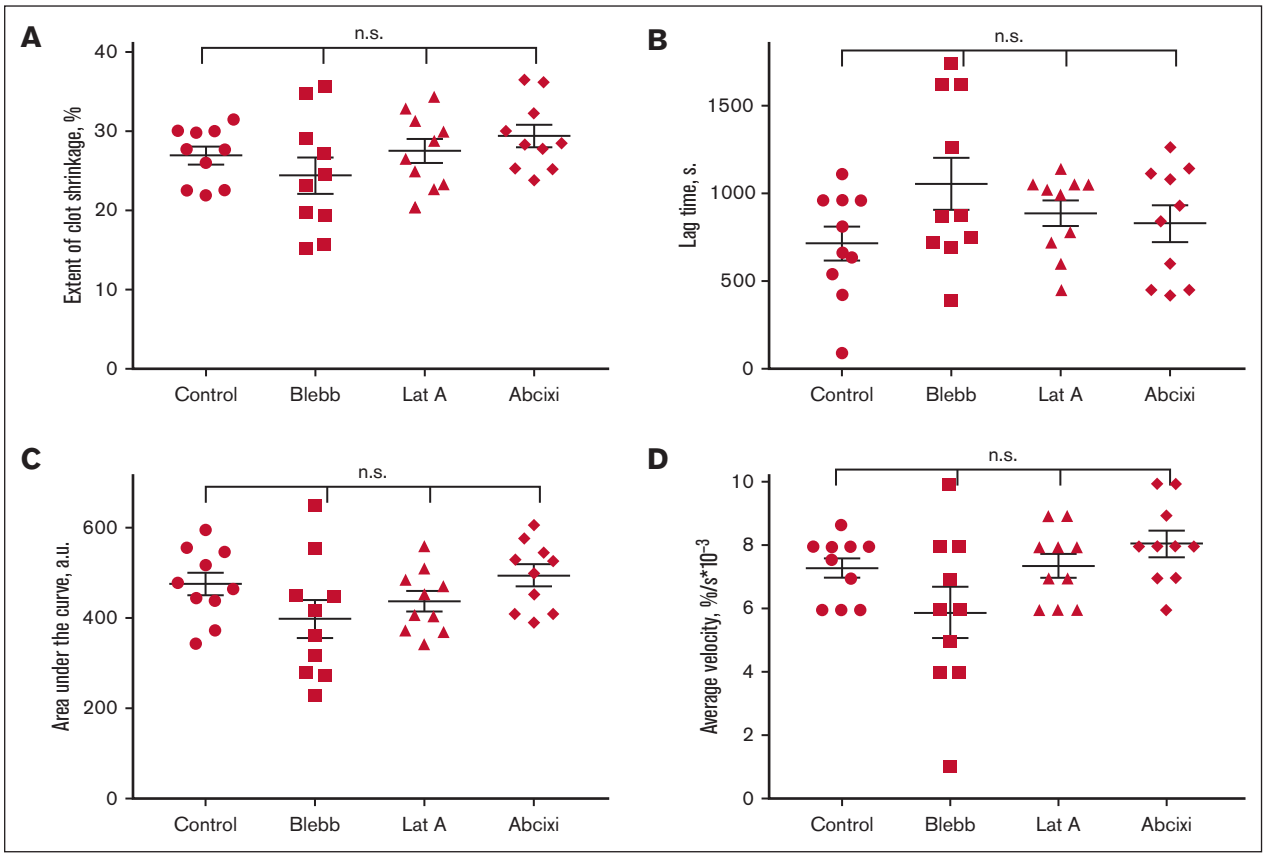

Figure 3. Parameters of RBC-induced clot shrinkage in platelet-depleted reconstituted blood samples in the absence (control) and presence of the following platelet inhibitors. 200 μM Blebb, 4 μM Lat A, and 100 μg/mL Abcixi. (A) The final extent of clot shrinkage. (B) Lag time of clot shrinkage after thrombin-induced clot formation. (C) Area under the kinetic curve. (D) Average shrinking velocity. The results indicate that the platelet antagonists studied have no effect on the RBC-induced clot shrinkage in the absence of platelets. The results from 5 duplicated experiments are presented as the mean ± standard error of the mean. Ordinary 1-way analysis of variance test with Tukey multiple comparisons post hoc test. Abcixi, abciximab; Blebb, blebbistatin; Lat A, latrunculin A; n.s., not significant.

$50\times10^3/\mu L$, and $70\times10^3/\mu L$), mimicking severe to moderate thrombocytopenia. Figure 2B shows a strong promoting effect of RBCs on the impaired clot contraction when the platelet number is low. The stimulating effect of RBCs was proportional to the background low platelet counts, suggesting that the RBCs enhance the reduced bulk platelet contractility in conditions resembling thrombocytopenia.

Structure and composition of clots formed from platelet-depleted reconstituted blood samples before and after the RBC-induced clot shrinkage

To get insights into the structural mechanisms of the RBC-induced clot shrinkage, we compared the morphology of the platelet-depleted reconstituted blood clots right after formation and after 1 hour of unconstrained compaction. As revealed by light microscopy and scanning electron microscopy, the initial clot contained relatively sparse mostly single RBCs (Figure 4A) that had a biconcave shape and were embedded randomly into a network of fibrin fibers (Figure 4C; supplemental Figure 8A,C). After the clot shrinkage, RBCs were arranged into aggregates (Figure 4B), either linear stacks (rouleaux) or unstructured 3D accumulations with the intercellular space filled with amorphous protein mass (Figure 4D; supplemental Figure 8B,D). Thus, the main structural difference between the platelet-depleted blood clots before and after shrinkage was the intercellular adhesion of RBCs, which suggests that the RBC aggregation is the driving force of clot compaction in the absence of platelets.

To see whether the observed RBC aggregation within a clot is because of fibrin formation, not other triggers, control experiments were performed in which RBCs were suspended and allowed to settle for 1 hour in PFP, thrombin-induced PRP-derived serum, and thrombin in the absence and presence of bovine serum albumin (supplemental Figure 9). When RBCs were suspended in PFP, minor macroscopic signs of RBC aggregation were observed (supplemental Figure 9Ai) confirmed by microscopically revealed small (<10 RBCs per aggregate) sporadic stacks of RBCs (supplemental Figure 9Aii). In the other environmental conditions studied, no signs of RBC aggregation were observed, either macro- or microscopically (supplemental Figure 9B-E). Therefore, the RBC aggregation inside a clot is triggered owing to the presence of fibrin, which entraps RBCs and brings them close together inside the network pores. No other components of plasma or serum, including active thrombin, were able to induce significant RBC aggregation by themselves, but serum proteins could augment the RBC aggregations via the osmotic depletion mechanism analyzed later.

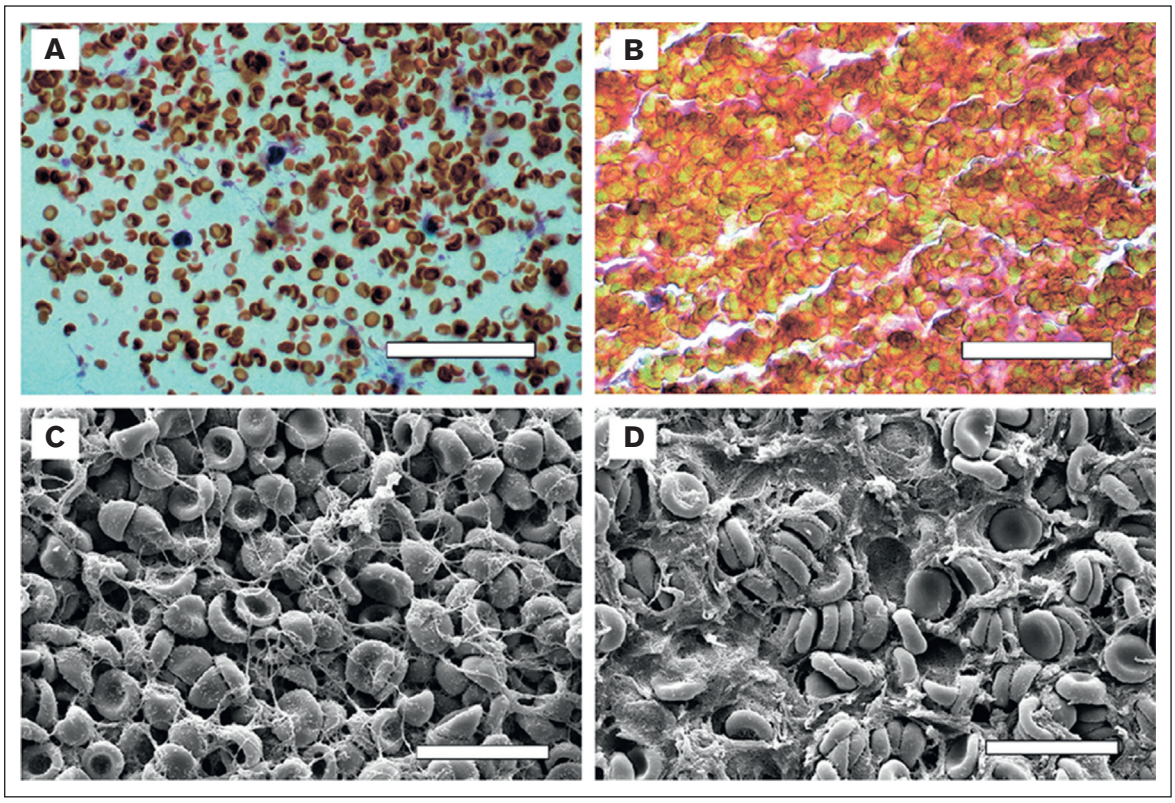

Figure 4. Morphology of the platelet-depleted blood clots right after formation and after unconstrained compaction. Representative light microscopy (A-B) and scanning electron microscopy (C-D) images of clots from platelet-depleted reconstituted blood samples before (A,C) and after (B,D) clot shrinkage over 60 minutes, showing nonaggregated (A,C) and aggregated (B,D) RBCs. Scale bars, 50 μm (A-B) and 15 μm (C-D).

Tension generated by the aggregating RBCs within platelet-depleted clots

Given that the RBC-induced compaction of a clot is a biomechanical process, it must have a measurable force that pulls on a fibrin network, making it more condensed. The average isometric tension generated by RBCs within a constrained, platelet-depleted clot in a rheometer was measured as 143 ± 121 mN. When normalized by the total number of RBCs in the sample, the estimated force per 1 RBC was ~120 ± 100 pN (Figure 5). This measurement reflects the aggregation forces produced by aggregating RBCs, which play a role in clot compaction in the absence of platelets.

Role of plasma proteins and depletion interactions in the RBC-induced clot shrinkage

To assess the necessity of plasma proteins in the RBC-induced clot shrinkage, we made clots from purified fibrinogen mixed with RBCs at a physiological count of ~4 × 10^6/μL. The results show that, in the absence of plasma proteins, RBCs did not induce shrinkage of a fibrin clot, at least within 1 hour of observation (Figure 6A), indicating that some plasma proteins, other than fibrinogen, are indispensable to the RBC aggregation and shrinkage of fibrin clots. This result also suggests that thrombin by itself is not a trigger of RBC aggregation because of the lack of RBC aggregation/agglutination in the presence of thrombin without fibrinogen (supplemental Figure 9). It is noteworthy that clot formation, either in plasma or in a purified system, results in fibrinogen to fibrin conversion without free fibrinogen left in the serum; therefore, the fibrinogen-mediated bridging mechanism for RBC aggregation within a clot seems highly unlikely.

To test whether the RBC-induced shrinkage of fibrin clots is osmotically driven, we added an osmotically active macromolecule, 100 kDa dextran, to the mixture of purified fibrinogen and platelet-depleted RBCs followed by clotting with thrombin. In the presence of dextran, the purified fibrin clots containing RBCs underwent apparent shrinkage (Figure 6A) and the effect was concentration dependent (Figure 6B-E). There was a clear dose-dependent increase in the extent of clot shrinkage (Figure 6C), shortening of the lag time (Figure 6D), and an increase in average velocity (Figure 6E) after 1 hour (supplemental Table 4). These results strongly suggest the importance of the osmotic depletion interactions between aggregating RBCs as a driving force of the RBC-induced shrinkage of fibrin clots.

As a negative control for the role of RBC aggregation in clot shrinkage, we performed experiments in platelet-depleted plasma mixed with RBCs rigidified by pretreating them with low concentrations of glutaraldehyde to reduce deformability and aggregability. Glutaraldehyde-treated RBCs had a significantly (on average of more than twofold) reduced ability to induce clot shrinkage (supplemental Figure 10A-D). This effect was because of the increased rigidity of glutaraldehyde-treated RBCs confirmed with ektacytometry (supplemental Figure 10E-H).

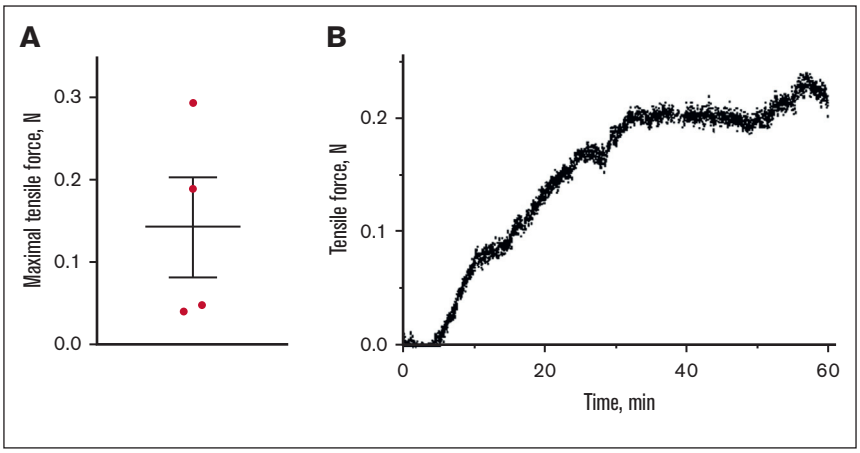

Figure 5. Tension generated by the aggregating RBCs within platelet-depleted clots measured with a rheometer. (A) Average maximal normal force generated by platelet-depleted reconstituted blood clots at 60 minutes after clot formation (mean ± standard error of the mean, n = 4 different donors). The RBC-related tension was determined as the difference between the maximal normal forces generated by the clots in the presence and absence of RBCs. (B) A representative tensile curve showing the dynamics of the tensile (normal) force generated by an RBC-containing platelet-depleted plasma clot formed between fixed horizontal plates of the rheometer.

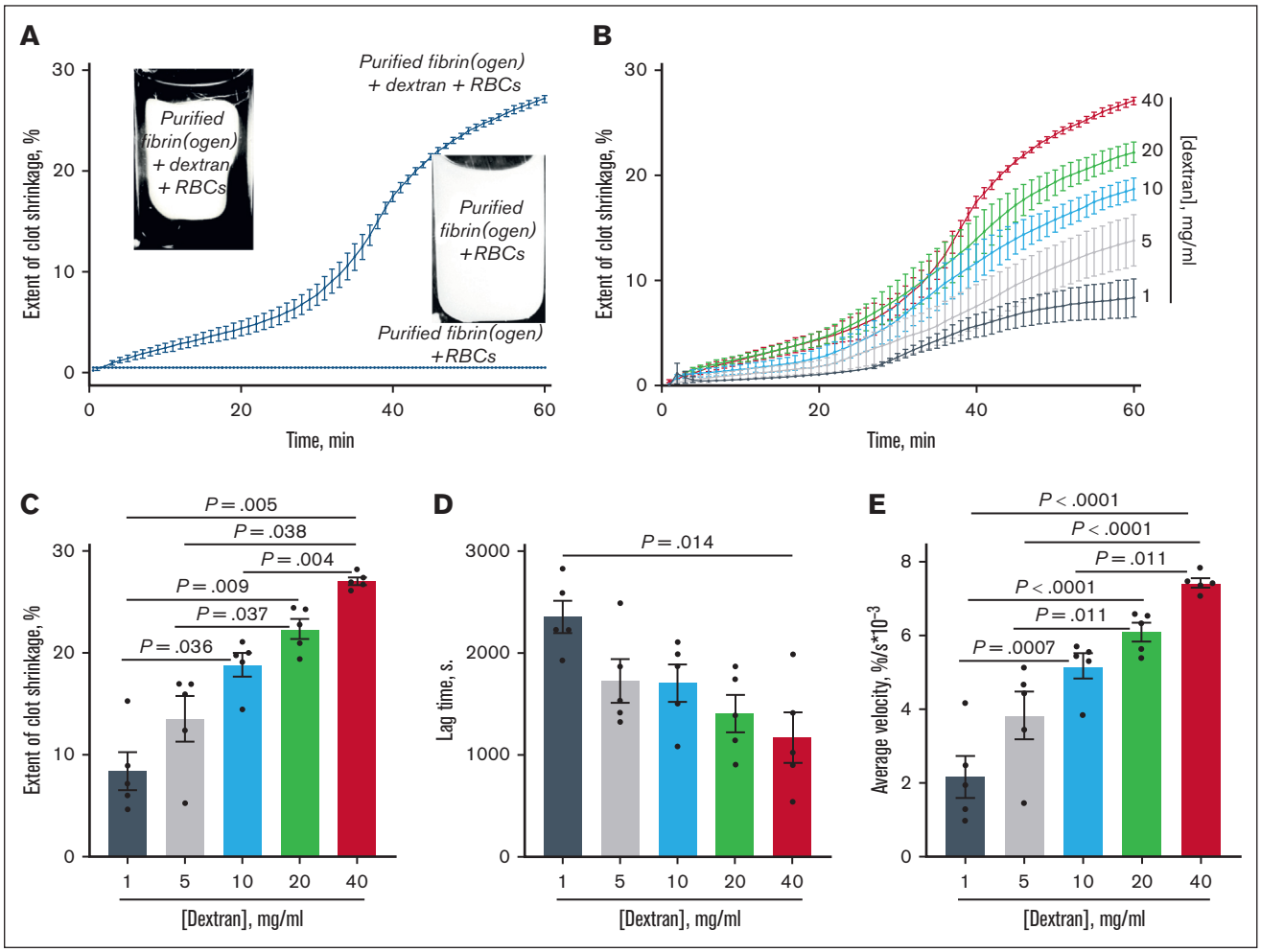

Figure 6. Kinetic curves and parameters characterizing the shrinkage of thrombin-induced clots made from purified fibrinogen mixed with washed RBCs in the absence and presence of various concentrations of 100 kDa dextran. (A) Comparative kinetic curves and representative photographic images showing a shrunken clot from purified fibrin(ogen) mixed with 100 kDa dextran and RBCs (left) vs an unshrunken clot from purified fibrin(ogen) mixed with RBCs only. (B) Kinetic curves of RBC-induced clot shrinkage (extent of clot shrinkage over time) at various dextran concentrations. (C) The extent of clot shrinkage after 1 hour. (D) Lag time of clot shrinkage after addition of thrombin. (E) Average shrinkage rate. The results from 5 duplicated independent experiments are presented as the mean ± standard error of the mean and analyzed with the ordinary 1-way analysis of variance test with Tukey multiple comparisons post hoc test.

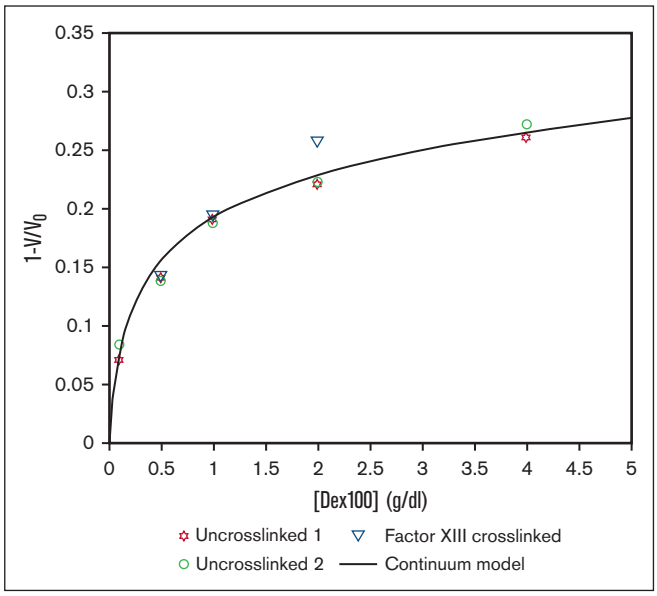

Figure 7. Equilibrium shrinkage of a fibrin clot containing RBCs in the presence of increasing concentrations of 100 kDa dextran. Prediction from continuum theory is shown together with experimental data for both cross-linked and uncross-linked fibrin clots containing RBCs. The overall trend of increasing contraction with increasing dextran concentration is captured by a theory that accounts for depletion and electrostatic interactions in addition to fibrin clot elasticity, RBC bridging interactions, and RBC-fibrin adhesion.

Effect of model parameters on clot shrinkage owing to RBC aggregation

A plot of the extent of contraction, $(1\text{-}V/V_0)$, as a function of dextran concentration c_2 at equilibrium is shown together with experimental data in Figure 7. The modeling parameters were consistent with measured characteristics of RBC interactions (supplemental Material, Section 3). The increased extent of contraction with an increase in dextran concentration is captured very well. We used the model to show how various parameters dictating the energetics of fibrin, RBC, and liquid govern the trends in clot shrinkage as a function of the osmolyte concentration. Supplemental Figure 11A shows that increasing the elastic modulus of the fibrin clot reduces clot shrinkage given that osmotic depletion forces encounter greater resistance to deforming the clots, whereas supplemental Figure 11B shows that changing the RBC-fibrin interactions by a similar multiple has a smaller effect. The latter suggests that the RBC-fibrin interactions play a weaker role than clot elasticity and RBC electrostatic interactions in clot shrinkage. The effects of various model parameters on clot shrinkage are discussed in detail in the supplemental Material, Section 3.

Discussion

Incorporation of RBCs into fibrin clots has important biological consequences, including alterations in fibrin network structure and clot mechanical properties,[25] predisposition to fracture,[44] lytic resistance,[45] and more. Here, we establish a hitherto unknown ability of RBCs embedded into a fibrin network to induce clot shrinkage or contraction.

In the presence of platelet inhibitors applied at highly effective concentrations, there is incomplete inhibition of the contraction process when clots are formed in whole blood, whereas the same inhibitors applied in PRP clots almost fully suppress contraction (Figure 1; supplemental Table 1). These observations have led us to the assumption that RBCs can play a mechanically active role in contraction of blood clots. To test this hypothesis, we have studied macroscopic thrombin-induced clots from reconstituted human blood samples made of PFP and platelet-depleted RBCs. These clots have no detectable platelets, yet they are seen to experience substantial clot shrinkage, exceeding 20% of the initial clot volume within an hour (Figure 2A). However, any active contractility of RBCs or other cells has been excluded because the clot shrinkage is insensitive to the inhibitors of nonmuscle myosin IIa (blebbistatin) and actin polymerization (latrunculin A) (Figure 3; supplemental Table 2), known to generate traction forces.[46,47] An alternative explanation for the mechanical activity of RBCs is their aggregation, which was confirmed morphologically in the shrunken clots containing RBCs (Figure 4). Moreover, we have shown directly the mechanical potential of aggregating RBCs and quantified the tensile force generated by the aggregated RBCs within a clot, which was found to be ~120 pN per 1 RBC, when the steady state is reached (Figure 5). Notably, this number is based on a unidirectional bulk force measurement and does not account for potential redistribution of tensile forces across the 3D structure of the clot. Nevertheless, this rough evaluation is consistent with an earlier study where the adhesion force between 2 RBCs was found to be in the range of 43 to 169 pN.[48] In addition, the mean adhesion force between 2 RBCs in the presence of lipopolysaccharide was determined to be 100 ± 84 pN.[49] Therefore, we have uncovered and quantified the mechanical interactions between aggregating RBCs that generate the forces driving the process of blood clot shrinkage. Theoretically, the fibrin gel may undergo spontaneous shrinkage known as gel syneresis,[50] but this pure physicochemical reaction can be excluded here, because there has been no fibrin clot shrinkage in the absence of RBCs (Figure 6A).

To create a more detailed mechanistic picture of the RBC-induced fibrin clot shrinkage, the experimental observations described earlier have been captured and analyzed in a new model for the mechanical behavior of clots containing fibrin and RBC (no platelets). The proposed model is a novel combination of a continuum gel model and a separate model for depletion interactions of RBCs, and it provides predictions about the extent of clot contraction as a function of osmolyte concentration in response to changes in RBC electrostatic interactions, fibrin network elasticity, RBC volume fraction, and strength of adhesive interactions between RBCs and fibrin (see the supplemental Material, Section 3; supplemental Figures 12-17). This continuum model takes account of fibrin gel poroelasticity, electrostatic, depletion, and bridging interactions of aggregated RBC and RBC-fibrin adhesive interactions. We computed the moduli corresponding to each of these interactions based on experimental data and found that bridging interactions of RBCs are relatively weak, RBC-fibrin adhesive interactions are stronger, and electrostatic and depletion interactions are strongest, but only when RBCs are in close proximity to each other. It is the formation of a fibrin network that provides a trigger for the aggregation of RBC by bringing them close together through embedment and constrained packing within network pores, so that electrostatic, depletion, and bridging interactions can come into

play. The aggregation of RBCs can cause shrinkage of a clot when the concentration of osmolyte (eg, dextran or plasma proteins) is high enough.[51] The osmotic depletion forces push the RBCs closer, whereas electrostatic repulsion drives them apart. Osmotic forces also work against the elasticity of the composite material formed by the fibrin network and aggregated RBCs interacting among themselves and fibrin. The forces owing to adhesion between RBC and fibrin help osmotic forces to bring RBCs closer together, but they are of much smaller magnitude. As such, the parameters controlling the electrostatic interactions of RBCs, the elasticity of the fibrin gel, and the adhesive interactions between RBCs and fibrin contribute to the physics of shrinkage of these clots, in addition to the concentration of the osmotic (oncotic) agents that control the depletion forces.

Based on our results and the literature on RBC aggregation, we postulate that there are 3 interconnected processes that result in the RBC-induced fibrin clot shrinkage. (1) After clot formation, RBCs get entrapped and brought close together within the pores of the fibrin network. (2) As a result of compaction, RBC aggregation occurs, driven by the osmotic gradient and depletion interactions, that is, a lower localized plasma protein concentration near the cell surface than the surrounding medium. (3) Owing to the RBC-fibrin binding interactions, the traction force generated during RBC aggregation is transmitted to fibrin fibers, causing shrinkage or contraction of the entire fibrin network.

The (patho)physiological importance of the revealed RBC-induced blood clot shrinkage is likely to be substantial in pathological conditions where the platelet-driven clot contraction is compromised. These conditions are related to many hereditary and acquired diseases associated with thrombocytopenia and/or thrombocytopathies.[52-62] This presumption has been confirmed in the experiments where the effects of RBCs on clot shrinkage were studied in the presence of low platelet counts, mimicking severe to moderate thrombocytopenia. The promoting effect of RBCs on the impaired clot contraction is quite substantial and proportional to the platelet counts, suggesting that the RBCs work in synergy with the platelet contractility, when it is not strong enough to effectively shrink a clot by itself (Figure 2B). These results strongly support the concept that, in case of weak bulk platelet contractility, RBCs at a physiological level can substantially improve clot contraction via a complementary or spare mechanism for clot compaction.

There are several other conceivable options that remain to be studied. RBC aggregation can normally contribute to the platelet-driven clot contraction of hemostatic clots and pathological thrombi. The extent of such contribution may be relatively small because the weaker RBC aggregation forces can be swamped out by the stronger contractile forces of activated platelets. In addition, RBC-induced shrinkage is likely to compete with platelet-driven contraction in venous thrombi with especially small amounts of platelets, such as chronic thrombi, thrombi in larger veins, or those associated with slower blood flow that may have a higher proportion of fibrin and RBCs. Notably, the volume fraction of platelets in venous thrombi is <1% compared with >60% for RBCs.[24] Finally, the contribution of RBCs to blood clot shrinkage is likely to be substantial when there are few and/or dysfunctional platelets in the blood in combination with high RBC counts, as in chronic myelogenous leukemia, myelofibrosis, polycythemia vera, and other myeloproliferative neoplasms,[63] as well as RBC transfusion in severe pancytopenia.[64]

In summary, this study provides compelling evidence that RBCs play a role in blood clot shrinkage unrelated to platelets. The main mechanism underlying the RBC aggregation within a clot is depletion interactions occurring when RBCs are embedded in close proximity to each other within the pores of the fibrin network. The potential pathophysiological significance of the RBC-induced clot shrinkage may be attributed to the ability of RBC aggregation to reinforce the platelet-driven blood clot contraction and/or promote clot compaction in thrombocytopenia and/or thrombocytopathies, especially associated with polycythemia, and in other pathological conditions with extremely low platelet content in thrombi. Certainly, the newly described phenomenon of RBC-mediated clot shrinkage needs further research to fully elucidate its (patho)physiological significance. As a part of these investigations, future studies must focus on biological and physical properties of RBC-shrunken clots, including permeability, sensitivity to fibrinolysis, mechanical properties, and much more.

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Authorship

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References

1. Cines DB, Lebedeva T, Nagaswami C, et al. Clot contraction: compression of erythrocytes into tightly packed polyhedra and redistribution of platelets and fibrin. *Blood*. 2014;123(10):1596-1603.
2. Cohen I. The mechanism of clot retraction. *Platelet Membr Glycoproteins*. 1985;17(2):299-323.
3. Litvinov RI, Weisel JW. Blood clot contraction: mechanisms, pathophysiology, and disease. *Res Pract Thromb Haemost*. 2023;7(1):100023.
4. Tutwiler V, Peshkova AD, Andrianova IA, Khasanova DR, Weisel JW, Litvinov RI. Contraction of blood clots is impaired in acute ischemic stroke. *Arterioscler Thromb Vasc Biol*. 2017;37(2):271-279.
5. Tutwiler V, Peshkova AD, Le Minh G, et al. Blood clot contraction differentially modulates internal and external fibrinolysis. *J Thromb Haemost*. 2019;17(2):361-370.
6. Peshkova A, Malyasyov D, Bredikhin R, et al. Reduced contraction of blood clots in venous thromboembolism is a potential thrombogenic and embologenic mechanism. *TH Open*. 2018;2(1):e104-e115.
7. Evtugina NG, Peshkova AD, Pichugin AA, Weisel JW, Litvinov RI. Impaired contraction of blood clots precedes and predicts postoperative venous thromboembolism. *Sci Rep*. 2020;10(1):18261.
8. Peshkova AD, Malyasev DV, Bredikhin RA, Giang LM, Litvinov RI. Contraction of blood clots is impaired in deep vein thrombosis. *Bionanoscience*. 2016;6(4):457-459.
9. Leong L, Chernysh IN, Xu Y, et al. Clot stability as a determinant of effective factor VIII replacement in hemophilia A. *Res Pract Thromb Haemost*. 2017;1(2):231-241.
10. Carr ME. Development of platelet contractile force as a research and clinical measure of platelet function. *Cell Biochem Biophys*. 2003;38(1):55-78.
11. Kim O V, Litvinov RI, Alber MS, Weisel JW. Quantitative structural mechanobiology of platelet-driven blood clot contraction. *Nat Commun*. 2017;8(1):1274.
12. Lam WA, Chaudhuri O, Crow A, et al. Mechanics and contraction dynamics of single platelets and implications for clot stiffening. *Nat Mater*. 2011;10(1):61-66.
13. Johnson GJ, Leis LA, Krumwiede MD, White JG. The critical role of myosin IIA in platelet internal contraction. *J Thromb Haemost*. 2007;5(7):1516-1529.
14. Höök P, Litvinov RI, Kim OV, et al. Strong binding of platelet integrin αIIbβ3 to fibrin clots: potential target to destabilize thrombi. *Sci Rep*. 2017;7(1):13001.
15. Tutwiler V, Mukhitov AR, Peshkova AD, et al. Shape changes of erythrocytes during blood clot contraction and the structure of polyhedrocytes. *Sci Rep*. 2018;8(1):17907.
16. Skorek P, Skubera M, Natorska J, Ząbczyk M, Smaś-Suska M, Tomkiewicz-Pająk L. The role of red blood cells in hemostasis and whole blood clot contraction in adults after the Fontan procedure. *Pol Arch Intern Med*. 2023;133(2):16412.
17. Sun Y, Le H, Lam WA, Alexeev A. Probing interactions of red blood cells and contracting fibrin platelet clots. *Biophys J*. 2023;122(21):4123-4134.
18. Tutwiler V, Litvinov RI, Lozhkin AP, et al. Kinetics and mechanics of clot contraction are governed by the molecular and cellular composition of the blood. *Blood*. 2016;127(1):149-159.
19. Tutwiler V, Wang H, Litvinov RI, Weisel JW, Shenoy VB. Interplay of platelet contractility and elasticity of fibrin/erythrocytes in blood clot retraction. *Biophys J*. 2017;112(4):714-723.
20. Evtugina NG, Peshkova AD, Khabirova AI, et al. Activation of Piezo1 channels in compressed red blood cells augments platelet-driven contraction of blood clots. *J Thromb Haemost*. 2023;21(9):2418-2429.
21. Tutwiler V, Litvinov RI, Protopopova A, et al. Pathologically stiff erythrocytes impede contraction of blood clots. *J Thromb Haemost*. 2021;19(8):1990-2001.
22. Faes C, Ilich A, Sotiaux A, et al. Red blood cells modulate structure and dynamics of venous clot formation in sickle cell disease. *Blood*. 2019;133(23):2529-2541.
23. Khismatullin RR, Nagaswami C, Shakirova AZ, et al. Quantitative morphology of cerebral thrombi related to intravital contraction and clinical features of ischemic stroke. *Stroke*. 2020;51(12):3640-3650.
24. Chernysh IN, Nagaswami C, Kosolapova S, et al. The distinctive structure and composition of arterial and venous thrombi and pulmonary emboli. *Sci Rep*. 2020;10(1):5112.
25. Gersh KC, Nagaswami C, Weisel JW. Fibrin network structure and clot mechanical properties are altered by incorporation of erythrocytes. *Thromb Haemost*. 2009;102(6):1169-1175.
26. Khismatullin RR, Abdullayeva S, Peshkova AD, et al. Extent of intravital contraction of arterial and venous thrombi and pulmonary emboli. *Blood Adv*. 2022;6(6):1708-1718.
27. Fåhraeus R. The suspension stability of the blood. *Physiol Rev*. 1929;9(2):241-274.
28. Bäumler H, Neu B, Donath E, Kiesewetter H. Basic phenomena of red blood cell rouleaux formation. *Biorheology*. 1999;36(5-6):439-442.
29. Wagner C, Steffen P, Svetina S. Aggregation of red blood cells: from rouleaux to clot formation. *C R Phys*. 2013;14(6):459-469.

30. Yu FTH, Armstrong JK, Tripette J, Meiselman HJ, Cloutier G. A local increase in red blood cell aggregation can trigger deep vein thrombosis: evidence based on quantitative cellular ultrasound imaging. *J Thromb Haemost*. 2011;9(3):481-488.
31. Litvinov RI, Weisel JW. Role of red blood cells in haemostasis and thrombosis. *ISBT Sci Ser*. 2017;12(1):176-183.
32. Brooks DE, Seaman GVF. The effect of neutral polymers on the electrokinetic potential of cells and other charged particles. I. Models for the zeta potential increase. *J Colloid Interf Sci*. 1973;43(3):670-686.
33. Maung Ye SS, Kim S. A mechanistic model of cross-bridge migration in RBC aggregation and disaggregation. *Front Bioeng Biotechnol*. 2022;10:1049878.
34. Chien S, Sung LA. Physicochemical basis and clinical implications of red cell aggregation. *Clin Hemorheol Microcirc*. 1987;7(1):71-91.
35. Asakura S, Oosawa F. On interaction between two bodies immersed in a solution of macromolecules. *J Chem Phys*. 1954;22(7):1255-1256.
36. Bäumler H, Neu B, Mitlöhner R, Georgieva R, Meiselman HJ, Kiesewetter H. Electrophoretic and aggregation behavior of bovine, horse and human red blood cells in plasma and in polymer solutions. *Biorheology*. 2001;38(1):39-51.
37. Spiewak R, Gosselin A, Merinov D, et al. Biomechanical origins of inherent tension in fibrin networks. *J Mech Behav Biomed Mater*. 2022;133:105328.
38. Garyfallogiannis K, Purohit PK, Bassani JL. Energy release rate for cracks in hydrogels undergoing finite deformations. *J Mech Phys Sol*. 2022;167:105009.
39. Sun MMC, Beam KS, Cerveny CG, et al. Reduction-alkylation strategies for the modification of specific monoclonal antibody bisulfides. *Bioconjug Chem*. 2005;16(5):1282-1290.
40. Brown AEX, Litvinov RI, Discher DE, Purohit PK, Weisel JW. Multiscale mechanics of fibrin polymer: gel stretching with protein unfolding and loss of water. *Science*. 2009;325(5941):741-744.
41. Lee K, Kinnunen M, Khokhlova MD, et al. Optical tweezers study of red blood cell aggregation and disaggregation in plasma and protein solutions. *J Biomed Opt*. 2016;21(3):035001.
42. López-Jaime F-J, Benítez O, Díaz Jordán BL, et al. Expert opinion paper on the treatment of hemophilia a with emicizumab. *Hematology*. 2023;28(1):2166334.
43. Neu B, Meiselman HJ. Depletion-mediated red blood cell aggregation in polymer solutions. *Biophys J*. 2002;83(5):2482-2490.
44. Fereidoonnezhad B, Dwivedi A, Johnson S, McCarthy R, McGarry P. Blood clot fracture properties are dependent on red blood cell and fibrin content. *Acta Biomater*. 2021;127:213-228.
45. Wohner N, Sótonyi P, MacHovich R, et al. Lytic resistance of fibrin containing red blood cells. *Arterioscler Thromb Vasc Biol*. 2011;31(10):2306-2313.
46. Kovács M, Tóth J, Hetényi C, Málnási-Csizmadia A, Sellers JR. Mechanism of blebbistatin inhibition of myosin II. *J Biol Chem*. 2004;279(34):35557-35563.
47. Coué M, Brenner SL, Spector I, Korn ED. Inhibition of actin polymerization by latrunculin A. *FEBS Lett*. 1987;213(2):316-318.
48. Steffen P, Verdier C, Wagner C. Quantification of depletion-induced adhesion of red blood cells. *Phys Rev Lett*. 2013;110(1):018102.
49. Steffen P, Jung A, Nguyen DB, et al. Stimulation of human red blood cells leads to Ca2+-mediated intercellular adhesion. *Cell Calcium*. 2011;50(1):54-61.
50. Pickering JW, Hewitt JA. The syneresis of blood clots. *Q J Exp Physiol*. 1923;13(3):199-207.
51. Pribush A, Zilberman-Kravits D, Meyerstein N. The mechanism of the dextran-induced red blood cell aggregation. *Eur Biophys J*. 2007;36(2):85-94.
52. Nurden P, Nurden AT. Congenital disorders associated with platelet dysfunctions. *Thromb Haemost*. 2008;99(2):253-263.
53. Israels SJ, Kahr WHA, Blanchette VS, Luban NLC, Rivard GE, Rand ML. Platelet disorders in children: a diagnostic approach. *Pediatr Blood Cancer*. 2011;56(6):975-983.
54. Bolton-Maggs PHB, Chalmers EA, Collins PW, et al. A review of inherited platelet disorders with guidelines for their management on behalf of the UKHCDO. *Br J Haematol*. 2006;135(5):603-633.
55. Alamelu J, Liesner R. Modern management of severe platelet function disorders. *Br J Haematol*. 2010;149(6):813-823.
56. Cox K, Price V, Kahr WH. Inherited platelet disorders: a clinical approach to diagnosis and management. *Expert Rev Hematol*. 2011;4(4):455-472.
57. Matthews DC. Inherited disorders of platelet function. *Pediatr Clin North Am*. 2013;60(6):1475-1488.
58. Kenny M, Pollitt AY, Patil S, et al. Contractility defects hinder glycoprotein VI-mediated platelet activation and affect platelet functions beyond clot contraction. *Res Pract Thromb Haemost*. 2024;8(1):102322.
59. Ono A, Westein E, Hsiao S, et al. Identification of a fibrin-independent platelet contractile mechanism regulating primary hemostasis and thrombus growth. *Blood*. 2008;112(1):90-99.
60. Flaumenhaft R, Dilks JR, Rozenvayn N, Monahan-Earley RA, Feng D, Dvorak AM. The actin cytoskeleton differentially regulates platelet α-granule and dense-granule secretion. *Blood*. 2005;105(10):3879-3887.
61. Handin RI. Inherited platelet disorders. *Hematology*. 2005;2005(1):396-402.
62. Williams MS, Coller BS, Väänänen HJ, Scudder LE, Sharma SK, Marmur JD. Activation of platelets in platelet-rich plasma by rotablation is speed-dependent and can be inhibited by abciximab (c7E3 Fab; ReoPro). *Circulation*. 1998;98(8):742-748.
63. Moliterno AR, Ratchford EV. Shedding new light on the magnitude of thrombosis risk in patients with myeloproliferative neoplasms. *Ann Intern Med*. 2018;168(5):363-364.
64. Jiang D, Houck KL, Murdiyarso L, et al. RBCs regulate platelet function and hemostasis under shear conditions through biophysical and biochemical means. *Blood*. 2024;144(14):1521-1531.