Lytic Resistance of Fibrin Containing Red Blood Cells

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Objective—Arterial thrombi contain variable amounts of red blood cells (RBCs), which interact with fibrinogen through an eptifibatide-sensitive receptor and modify the structure of fibrin. In this study, we evaluated the modulator role of RBCs in the lytic susceptibility of fibrin.

Methods and Results—If fibrin is formed at increasing RBC counts, scanning electron microscopy evidenced a decrease in fiber diameter from 150 to 96 nm at 40% (v/v) RBCs, an effect susceptible to eptifibatide inhibition (restoring 140 nm diameter). RBCs prolonged the lysis time in a homogeneous-phase fibrinolytic assay with tissue plasminogen activator (tPA) by up to 22.7±1.6%, but not in the presence of eptifibatide. Confocal laser microscopy using green fluorescent protein–labeled tPA and orange fluorescent fibrin showed that 20% to 40% (v/v) RBCs significantly slowed down the dissolution of the clots. The fluorescent tPA variant did not accumulate on the surface of fibrin containing RBCs at any cell count above 10%. The presence of RBCs in the clot suppressed the tPA-induced plasminogen activation, resulting in 45% less plasmin generated after 30 minutes of activation at 40% (v/v) RBCs.

Conclusion—RBCs confer lytic resistance to fibrin resulting from modified fibrin structure and impaired plasminogen activation through a mechanism that involves eptifibatide-sensitive fibrinogen-RBC interactions. (***Arterioscler Thromb Vasc Biol*. 2011;31:2306-2313.)**

Key Words: blood cells ■ fibrin ■ fibrinolysis ■ plasminogen activators ■ platelet receptor blockers

A reverse correlation between bleeding time and red blood cell (RBC) count was observed more than a century ago.[1] When anemic and thrombocytopenic patients were treated with transfusion, the correction of the bleeding time correlated with the corrected hematocrit despite the fall in the platelet count to pretransfusion levels. Later studies[2] confirmed that the prolonged bleeding time of severely anemic patients can be corrected with washed RBC transfusion (thus excluding the effect of plasma factors) at essentially unchanged platelet counts. The mechanism of RBC contribution to hemostatic function is still open to question, notwithstanding their known role in the maintenance of blood viscosity, the chemical signaling of platelet activation, and the provision of phospholipid support for the activation of coagulation factors (reviewed by Wohner[3]). The solid matrix of hemostatic and pathological blood clots is composed of fibrin and variable cellular elements, but the incorporation of RBCs appears to be mediated through a less specific entrapment than that of platelets.[4] Based on binding data, approximately 2% of fibrinogen, the plasma precursor of fibrin monomers, is estimated to circulate in vivo in association with erythrocytes.[5] This interaction is not simply a nonspecific protein adhesion to the cell membrane, but it involves an erythrocyte receptor[6] and a specific domain around residues 207 to 303 of fibrinogen Aα-chains.[7] Recently, the RBC receptor for fibrinogen has been identified as an integrin related to the platelet $\alpha_{IIb}\beta_3$ receptor with similar eptifibatide sensitivity and impaired function in Glanzmann thrombasthenia.[8] When fibrinogen is converted to fibrin, the presence of RBCs modifies the structural and viscoelastic characteristics of plasma clots.[9,10] Because fibrin structure profoundly affects the subsequent removal of blood clots from the vasculature by tissue plasminogen activator (tPA)–dependent proteolysis as a basic fibrinolytic mechanism (reviewed recently by Weisel and Litvinov[11] and Lord[12]), the presence of RBCs may change the lytic susceptibility of thrombi, but no direct evidence for such modulation of fibrin dissolution is currently available. The present study was undertaken in an attempt to characterize the impact of RBCs on 2 distinct stages of the fibrinolytic process, plasminogen activation by tPA and fibrin degradation by the generated plasmin, and correlate it to the observed structural changes caused by the incorporation of RBCs in pure fibrin clots.

Methods

Isolation and Labeling of RBCs

RBCs were isolated from citrated whole blood collected from healthy volunteers with venipuncture. Within 1 hour after collection,

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platelet-rich plasma was removed following centrifugation at 150 *g* for 10 minutes, and the cell pellet was further centrifuged at 2000 *g* for 10 minutes to sediment the RBCs. The RBC pellet was washed 3 times with 10 volumes of 1.5 mmol/L KH_2PO_4, 8.1 mmol/L Na_2HPO_4 buffer, pH 7.4, containing 137 mmol/L NaCl, 2.7 mmol/L KCl, and 5 mmol/L glucose (PBS) by resuspension and centrifugation at 1200 *g* for 5 minutes. RBCs were counted using an Abacus Junior B hematology analyzer (Diatron GmbH, Vienna, Austria), and the hematocrit of the RBC suspension was adjusted to 0.8 with PBS. RBCs were stored for up to 2 hours at room temperature before use. For the measurements using confocal laser microscopy, washed RBCs were labeled with Vybrant DiD cell-labeling solution (Invitrogen Life Technologies, Budapest, Hungary) by mixing 5 mL of RBC suspension diluted to a hematocrit of 0.016 in PBS with 25 μL of Vybrant DiD solution for 30 minutes followed by 3 wash cycles (resuspension/centrifugation at 1200 *g* for 5 minutes) with PBS. On the basis of the cell count, the hematocrit of the Vybrant-labeled RBCs in the final pellet was adjusted to 0.8.

Ball Sedimentation Assay of Fibrin Dissolution

The assay was designed as an alternative to the reference method from the European Pharmacopoeia for determination of tPA potency,[13] adapted for the high opacity of clots containing RBCs. Two experimental setups were used, in both of which the total amount of fibrin in the clot was constant, but in Model 1 this was achieved with increasing extracellular concentrations of fibrinogen (from 7.4 to 12.4 μmol/L human fibrinogen, plasminogen depleted, Calbiochem, La Jolla, CA) compensating for the rising RBC occupancy (from 0 to 40% [v/v]) in a total volume of 2 mL, whereas in Model 2, the extracellular concentration of fibrinogen was constant (7.4 μmol/L) in a clot volume increasing from 0.8 mL in the absence of RBCs up to 1.35 mL at 40% (v/v) RBCs. The RBC-fibrinogen mixtures were supplemented with varying amounts of plasminogen isolated from human plasma,[14] $CaCl_2$, and in certain cases eptifibatide (GlaxoSmithKline Kft, Budapest, Hungary), so that the final extracellular concentrations in the assay clots were always 0.1 μmol/L plasminogen, 1 mmol/L $CaCl_2$, and 20 μmol/L eptifibatide. For all assays, thrombin of low specific activity (Serva, Heidelberg, Germany) was further purified by ion-exchange chromatography on sulfopropyl-Sephadex, yielding a preparation with a specific activity of 2100 IU/mg,[15] and 1 IU/mL was considered equivalent to approximately 10.7 nmol/L by active site titration.[16] Clotting and fibrinolysis were initiated simultaneously in transparent reaction tubes with a diameter of 0.8 cm by mixing RBC-fibrinogen suspensions with PBS containing thrombin and tPA (Boehringer Ingelheim, Ingelheim, Germany) at concentrations needed to reach final extracellular values of 85 and 1 nmol/L, respectively. After 15 seconds, a steel ball with a diameter of 2 mm and a weight of 0.13 g was placed on the surface of the clot, and the time elapsed until the ball reached the bottom of the tube (lysis time) was measured as an indicator of the collapse of identical quantities of fibrin.

Plasminogen Activation Assays

For the plasminogen activation assay on the surface of the clot, RBC suspensions in fibrinogen were prepared as described above for the fibrinolytic assay (only plasminogen was applied at a different concentration, so that the final extracellular value in the clots was always 0.5 μmol/L). The RBC-fibrinogen mixtures were clotted with 16 nmol/L thrombin in a total volume of 75 μL in 96-well microplates for 30 minutes. Thereafter, 150 μL of 15 nmol/L tPA was added to the surface of the clots, and after 30 minutes the fluid phase was removed in ice-cold tubes and centrifuged at 2000 *g* for 10 minutes at 4°C to remove cell debris. The plasmin activity in the supernatant was determined by mixing 100 μL of supernatant and 100 μL of 0.2 mmol/L Spectrozyme-PL (H-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide, American Diagnostica, Pfungstadt, Germany) and measuring absorbance at 405 nm (activity was expressed in ΔA_{405}/min). To evaluate the effect of eptifibatide on plasminogen activation in the absence of fibrin, a 2-stage activation assay was used, as previously described.[17] Briefly, 3 μmol/L plasminogen containing no eptifibatide or 20 μmol/L eptifibatide was mixed with 70 nmol/L tPA, samples were taken at intervals, and the amidolytic activity of the generated plasmin was measured using 0.1 mmol/L Spectrozyme-PL.

Expression of Fluorescent Chimeric tPA Variant (tPA-GFP)

Recombinant human tPA-jellyfish green fluorescent protein (GFP) was constructed and expressed using the Bac-to-Bac baculovirus expression system as a tPA-C-terminal fusion with enhanced GFP isolated from the pEGFP plasmid (Clontech, Mountain View, CA), as described previously.[18,19]

Confocal Microscopic Imaging in the Course of Fibrinolysis

RBC suspensions in fibrinogen were prepared as described for the plasminogen activation assay using Vybrant-labeled RBCs and replacing 1% of the fibrinogen with Alexa Fluor 546–conjugated fibrinogen (Invitrogen Life Technologies, Budapest, Hungary). Fibrinogen was clotted with 16 nmol/L thrombin for 30 minutes in 0.5-mm-high chambers constructed from glass slides. Thereafter, 60 nmol/L tPA-GFP was added to the edge of the clot, and the fluorescence (excitation wavelength 488 nm, emission wavelength 525 nm for tPA-GFP detection; excitation wavelength 543 nm, emission wavelength 575 nm for Alexa Fluor 546–fibrinogen detection; excitation wavelength 633 nm, emission wavelength 650 nm for Vybrant-labeled RBC detection) was monitored with Confocal Laser Scanning System LSM510 (Carl Zeiss GmbH, Jena, Germany) taking sequential images of the fluid-fibrin interface at a distance of approximately 50 μm from the glass surface with identical exposures and laser intensities using a Plan-Neofluar ×20/0.5 objective.

Scanning Electron Microscope Imaging of Thrombi and Fibrin

The RBC-fibrinogen mixtures were clotted with 16 nmol/L thrombin in a total volume of 100 μL, and after 30 minutes clots were placed into 10 mL of 100 mmol/L Na-cacodylate, pH 7.2, buffer for 24 hours at 4°C. Following repeated washes with the same buffer, samples were fixed in 1% (v/v) glutaraldehyde for 16 hours. The fixed samples were dehydrated in a series of ethanol dilutions (20 to 96% [v/v]), a 1:1 mixture of 96% (v/v) ethanol/acetone, and pure acetone, followed by critical point drying with CO_2 in a E3000 Critical Point Drying Apparatus (Quorum Technologies, Newhaven, United Kingdom). The specimens were mounted on adhesive carbon discs and sputter coated with gold using a SC7620 Sputter Coater (Quorum Technologies), and images were taken with an EVO40 scanning electron microscope (Carl Zeiss GmbH).

Morphometric Analysis of Fibrin Structure and Statistical Procedures

The scanning electron microscope images of thrombi and fibrin were analyzed to determine the diameter of the fibrin fibers using self-designed scripts running under the Image Processing Toolbox, version 7.0, of Matlab 7.10.0.499 (R2010a) (Mathworks, Natick, MA). For the diameter measurements, a grid was drawn over the image with 10 to 15 equally spaced horizontal lines, and all fibers crossed by them were included in the analysis. The diameters were measured by manually placing the pointer of the Distance tool over the end points of transverse cross-sections of 300 fibers from each image (always perpendicularly to the longitudinal axis of the fibers). The distribution of the data on fiber diameter was analyzed using an algorithm described previously for identification of a theoretical distribution that gives the best global fit to several empirical data sets.[20,21] The best-fitted distributions for different samples were compared using the Kuiper test, and probability values were calculated with Monte Carlo simulation procedures. When a statistically significant difference between 2 distributions was established,[20] the numeric characteristics of the central tendency and variance were considered to be statistically significant. The statistical hypothesis

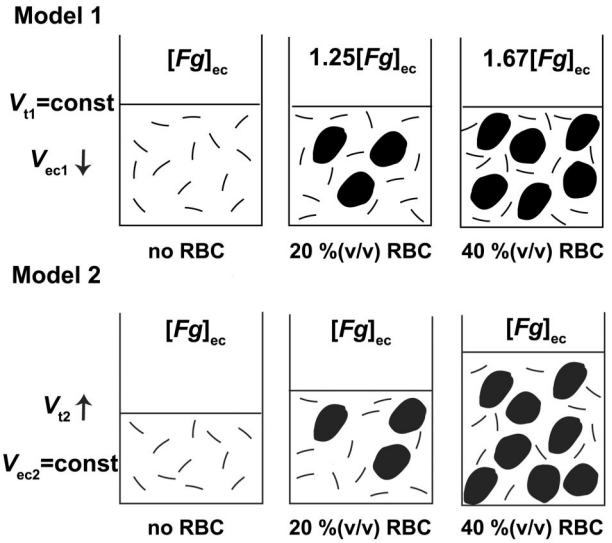

Figure 1. Schemes of the experimental setups used in the evaluation of fibrinolysis in the presence of red blood cells (RBCs). The total volume (V_t) of the clot was partially occupied by RBCs, whereas fibrinogen (converted to fibrin) occupied the extracellular volume (V_{ec}) at concentration $[Fg]_{ec}$. In both models, the total amount of fibrin ($V_{ec} \cdot [Fg]_{ec}$) was identical at any RBC occupancy, which was maintained by the indicated increase in $[Fg]_{ec}$ at decreasing V_{ec} in Model 1 or at constant $[Fg]_{ec}$ and V_{ec} in Model 2. Because in Model 1 two variables (fibrinogen concentration and RBC occupancy) were changed in parallel, separate cell-free clots were also prepared at identical fibrinogen concentrations and used as a reference for each RBC occupancy to isolate the RBC effects in the experiments performed according to this model.

testing for differences in other experimental measurements in this report was performed with the Kolmogorov-Smirnov test (Statistical Toolbox 7.3 of Matlab).

Results

Thrombi formed in vivo under similar rheological conditions in large arteries showed remarkable differences in their RBC content (Supplemental Figure I, available online at http://atvb.ahajournals.org). Even within a single thrombus, regions of high and low RBC count could be identified indicating the prevalence of local factors (blood vessel geometry, flow pattern) as determinants of thrombus composition. Thus, a correct understanding of the impact of RBCs on the lytic susceptibility of such compartmental architecture should be based on data modeling the structure and lysis of fibrin over a range of relevant RBC counts. The present study was restricted to clots formed from purified fibrinogen and washed RBCs. Because of the known compartmentalization of fibrin and cellular components within thrombi,[10,22] 2 extreme model states were evaluated (Figure 1). At increasing RBC occupancy, identical amounts of fibrin were present either in clots with constant total volume (and consequently increasing extracellular fibrin concentration; Model 1) or in clots with volume expanding proportionally to the increase of RBC content (preserving constant extracellular fibrin concentration; Model 2). In all cases, the intercellular fibrin was composed of thinner fibers and smaller pores, resulting in a denser network compared with the cell-free fibrin, as illustrated for Model 1 in Figure 2 (Supplemental Figure II).

The role of the integrin-dependent RBC-fibrinogen interaction in the modification of fibrin structure was approached with the addition of the integrin blocker eptifibatide (Figure 2A) at a concentration that has been shown to be efficient in the inhibition of fibrinogen binding to RBCs.[8] Because the most striking changes in the fibrin structure related to the presence of RBCs and eptifibatide appeared to be in the fiber diameter, this parameter was quantitatively evaluated using the morphometric approach illustrated in Figure 2B. In all cases, the increase in the clot occupancy by RBCs was coupled to a decrease in the fiber diameter (Table). This effect was more pronounced in Model 1 (at increasing extracellular fibrinogen concentrations), where 40% (v/v) RBC occupancy resulted in a more than 2-fold diameter

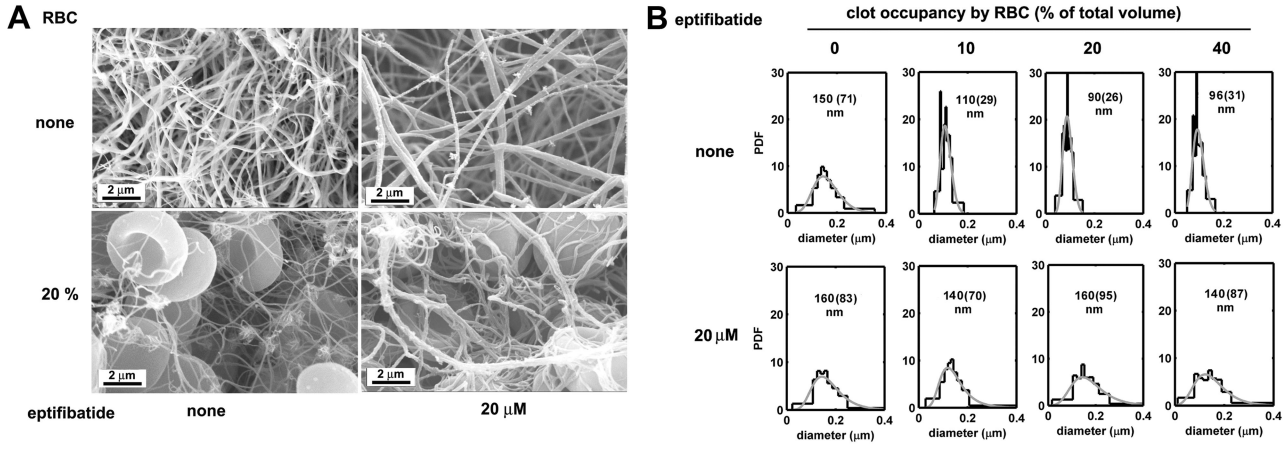

Figure 2. Changes in the fibrin network structure caused by red blood cells (RBCs) and eptifibatide. The scanning electron microscope images in A illustrate the fibrin structure in clots of identical volume and fibrinogen content in the absence or presence of 20% RBCs (note that expulsion of fibrinogen by RBCs increases the extracellular fibrinogen concentration in this model). B, Fiber diameter measured from the scanning electron microscope images for a range of RBC occupancy in the same clot model. Probability density functions (PDF) of the empirical distribution (black histogram) and the fitted lognormal theoretical distribution (gray curves) are presented with indication of the median and the interquartile range (in parentheses) of the fitted theoretical distributions. In the presence of RBCs, the parameters of the fitted distributions of the eptifibatide-free and eptifibatide-treated fibers differ at the $P<0.001$ level (for the RBC-free fibrins, the eptifibatide-related difference is not significant, $P>0.05$). Four replicate samples of each clot type were evaluated in a single global statistical procedure.

Table. Fiber Diameter (nm) of Fibrin Clots With Varying Red Blood Cell Content

	No Eptifibatide			20 μmol/L Eptifibatide		
	Model 1		Model 2	Model 1		Model 2
Volume Occupancy	+RBC	Reference	+RBC	+RBC	Reference	+RBC
10%	110† (29)	170† (91)	160† (90)	140† (70)	170† (86)	170 (83)
20%	90* (26)	170 (99)	100* (50)	160* (95)	180 (86)	180* (88)
40%	96* (31)	200* (100)	92* (33)	140* (87)	210* (110)	170 (86)

Fibrin was prepared and the scanning electron microscope (SEM) images were analyzed as illustrated in Figure 2. The median and interquartile range (in parentheses) of the diameter distributions are presented in nm units (4 replicate samples of each clot type were evaluated in a single statistical procedure). Reference values refer to cell-free fibrin prepared from fibrinogen at concentrations equivalent to the extracellular concentrations resulting from the expulsion of fibrinogen by the respective red blood cell (RBC) occupancy as described for Model 1 in Methods (8.2, 9.2, and 12.4 μmol/L in increasing order of RBC occupancy). For Model 2, the extracellular concentration of fibrinogen was always 7.4 μmol/L, and thus a single reference value of 150 (71) nm was used in the absence of eptifibatide and 160 (83) nm in the presence of 20 μmol/L eptifibatide. All differences between fiber diameters in the presence of RBCs and the respective reference values in Model 1 are significant at the $P<0.001$ level.

*,†Additional significant ($P<0.001$) differences are also indicated between two subsequent values along the columns from top to bottom (*) or in comparison with the cell-free 7.4 μmol/L fibrin (†).

reduction. On the basis of these results, we propose that in Model 1 the fiber diameter reflects the outcome of 2 opposing effects: the fiber-thickening effects of increasing fibrinogen concentrations in the absence of RBCs (reference values in column 2 of the Table) and the fiber-thinning effect of increasing RBC occupancy at constant fibrinogen concentration (Model 2, Table). Although it had no effect on the architecture of clots formed from purified fibrinogen, eptifibatide at 20 μmol/L completely reversed the changes in fiber diameter caused by RBCs in Model 2, supporting the role of the specific RBC receptor-fibrinogen interaction in the assembly of the final fibrin structure. In Model 1, the influence of eptifibatide was less pronounced, resulting in up to 40% reduction in the fiber-thinning effect of RBCs, probably related to the stronger competition on behalf of the increasing fibrinogen concentration in this experimental setup.

When fibrinogen containing plasminogen is mixed with thrombin and tPA, following clotting the plasminogen activator is uniformly dispersed in the total volume of the clot and the generated plasmin dissolves the fibrin matrix. As a result of proteolysis, fibrin loses its mechanical stability, and thus a steel ball placed on the top of the clot will descend in response to gravity. This global lytic assay system is intimately related to the biological function of fibrin, because it reflects the changes in the mechanical stability of the fibrin matrix. The time to achieve complete collapse of the clot can be used as a global end-point indicator of this assay. If identical quantities of fibrin are degraded, this lysis time depends on the combined effect of several factors (stability of the initial fibrin network, rate of plasminogen activation, catalytic efficiency of plasmin in various fibrin structures). Although the constant amount of fibrin was maintained differently in our Models 1 and 2 defined above, in both situations the presence of 10% and 40% (v/v) RBCs slowed down the fibrinolysis induced by incorporated tPA, and eptifibatide partially or completely reversed the RBC effect (Figure 3). In view of our recent data on the differential impact of fibrin structure on plasminogen activation and fibrin dissolution,[19] the discordant response of the 20% (v/v) RBC-fibrin clots in this complex fibrinolytic assay indicated the necessity to dissect the role of RBCs in these 2 stages of the process.

When tPA was applied to the surface of fibrin containing plasminogen and increasing numbers of RBCs, plasmin was generated on a fibrin surface with decreasing area (part of the interface was occupied by RBCs) and varying structure (Figure 2 and the Table), which is also known to affect plasminogen activation.[19] Under the conditions of Model 1, after a 30-minute activation on the surface of fibrin containing 10% to 20% (v/v) RBCs, the plasmin level peaked at 36.6% to 44.6% over the cell-free control values, but further increase in the RBC content moderated this stimulation to 25.6% at 40% (v/v) RBCs (Figure 4A). It is noteworthy that on the surface of cell-free fibrin formed from fibrinogen at concentrations equivalent to the extracellular values in Model 1, plasmin generation decreased with increasing fibrinogen concentrations (22.2% less plasmin formed at 12.4 μmol/L fibrinogen than at 7.4 μmol/L; data not shown). In contrast to the bell-shaped dependence of plasminogen activation on the RBC count in Model 1, a linear decrease in the amount of plasmin generated could be observed at increasing RBC occupancy and constant extracellular fibrinogen concentration (Figure 4B). Because the RBC-occupied area of the activation interface was the same in both models for identical volume occupancy, the differences in plasmin generation can be attributed to the variations in fibrin structure. In line with this, eptifibatide at 20 μmol/L moderated the effect of the highest RBC counts in both models of fibrin-dependent plasminogen activation (in the absence of fibrin, eptifibatide modestly stimulated the tPA-induced plasminogen activation, resulting in 20% more plasmin at 30 minutes; data not shown).

The interplay of tPA, fibrin, and RBCs at the fluid phase–clot interface was approached with confocal microscopic observation of tPA-GFP penetration in the clot, propagation of the lytic front of orange fluorescent–labeled fibrin, and release of fluorescent-labeled RBCs from the clot. In agreement of our and others' earlier findings,[19,23] within 10 minutes after

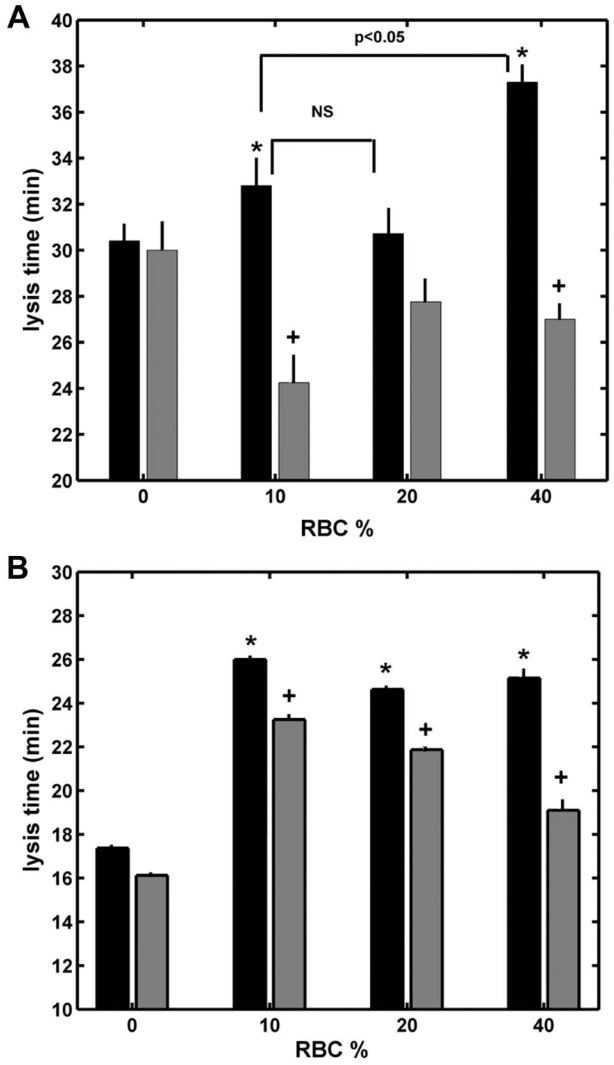

Figure 3. Fibrinolysis induced by clot-incorporated tissue plasminogen activator (tPA) in the presence of red blood cells. Clots of various red blood cell (RBC) occupancy contained 5 mg of fibrin at constant volume and increasing extracellular concentrations (A) or 2 mg of fibrin at increasing volumes and constant extracellular concentration (B), as well as plasminogen and tPA at identical extracellular concentrations as detailed in Methods. After 15 seconds of clotting, a steel ball was placed on the surface of the clots, and the lysis time was measured in the absence (black columns) or presence (gray columns) of eptifibatide. Data are presented as mean and SD ($n=4$). *Significant ($P<0.05$) difference between the fibrin without RBCs. +Significant ($P<0.05$) difference between eptifibatide-free and eptifibatide-treated fibrins with identical RBC content.

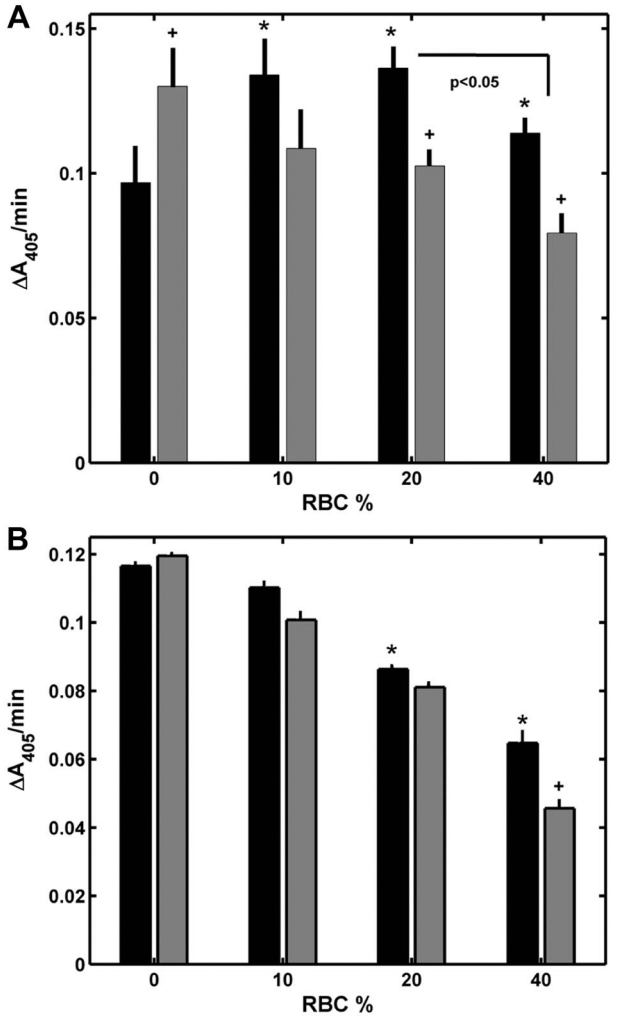

Figure 4. Plasminogen activation on the surface of fibrin containing red blood cells (RBCs). Plasmin activity (ΔA_{405}/min) was measured after 30 minutes of activation by tissue plasminogen activator (tPA) applied to the surface of clots with embedded plasminogen as described in Methods. The same RBC-fibrin model clots were used as in Figure 3 (A, varying extracellular fibrinogen concentration; B, constant fibrinogen concentration). In A, only the cell-free control for 9.2 μmol/L fibrin concentration, corresponding to 20% RBC clot occupancy, is shown, whereas in B, the control refers to 7.4 μmol/L fibrin. Data are presented as mean and SD ($n=4$) in the absence (black columns) or presence (gray columns) of 20 μmol/L eptifibatide. *Significant ($P<0.05$) difference between the fibrin without RBCs. +Significant ($P<0.05$) difference between eptifibatide-free and eptifibatide-treated fibrins with identical RBC content.

application of tPA-GFP to the surface of preformed fibrin, a sharp zone of intense tPA-related fluorescence was formed on the surface of cell-free fibrin (Figure 5A) and preserved throughout the lysis observation period (Figure 5B). In contrast, the presence of RBCs prevented this accumulation of tPA (Figure 5A and 5B). The progress of fibrin dissolution could be monitored on the basis of the position of the boundary layer of the fluorescent fibrin phase (Figure 5C). The presence of RBCs at 20% to 40% in the clot significantly slowed down the dissolution of fibrin after a 25-minute exposure to tPA, with the greatest difference at 30 minutes (3.5-fold shorter distance run by the lytic front), followed by a catch-up phase, but the difference remaining almost 2-fold at 55 minutes. Eptifibatide did not affect the progress of lysis in the absence of RBCs, but it moderated the inhibiting effect of RBCs. The release of RBCs from the clot occurred in parallel with the progress of the lytic process, as evidenced by the lack of RBCs in the fluid phase in the early stage of dissolution (Figure 5A) and their abundance out of the clot at later stages (Figure 5B).

Discussion

The hemostatic function of whole blood is a delicate balance of coagulation and fibrinolytic pathways, which is profoundly

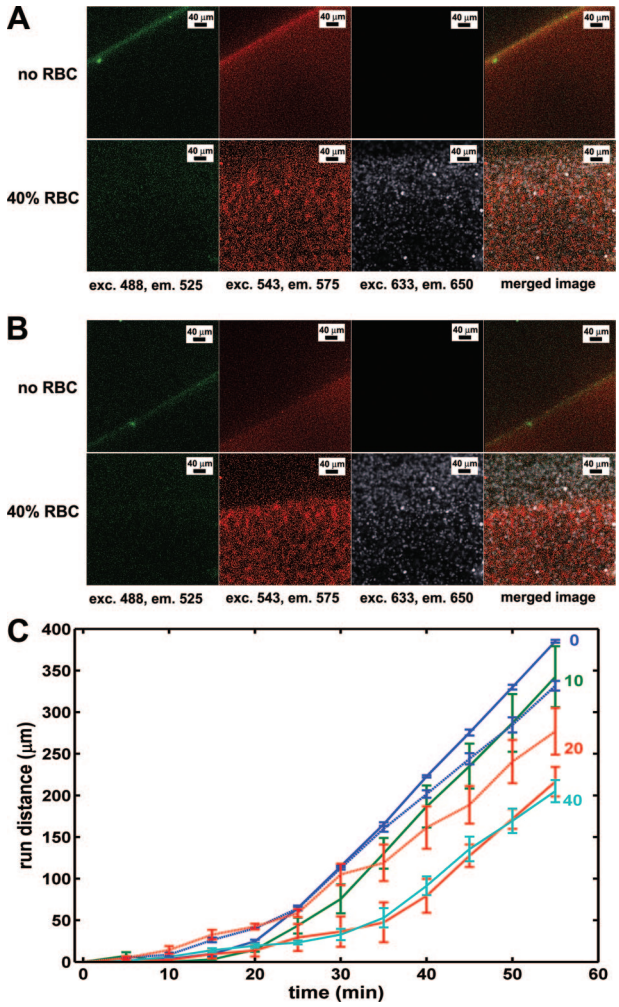

Figure 5. Effect of red blood cells on fibrinolysis induced by tissue plasminogen activator (tPA) applied to the surface of clots. Fibrin clots were prepared from fibrinogen containing Alexa Fluor 546–labeled fibrinogen, Vybrant DiD–labeled red blood cells (RBCs), and plasminogen. Following clotting, tPA-green fluorescent protein (GFP) was added to fibrin, and the fluid-fibrin interface was monitored by confocal laser scanning microscopy using triple fluorescent tracing at the indicated wavelengths. The images were taken 5 (A) and 40 (B) minutes after the addition of tPA-GFP (the tPA signal is shown in green, the fibrin in red, and the RBCs in white). C, Distance run by the fibrin boundary layer in the course of lysis (mean and SD of 3 measurements). The numbers next to the curves in the same color indicate the volume occupancy by RBCs. In some experiments, fibrin contained 20 μmol/L eptifibatide (dashed lines).

affected by RBCs. Although severely anemic patients show a bleeding trend, which can be reversed with transfusion of washed RBCs,[2] the increased RBC mass of polycythemia vera patients is coupled to frequent (in more than one third of the patients) thrombotic complications.[24] However, the local number of RBCs in thrombi does not necessarily correlate with the systemic RBC count, because some complicating factors, such as flow conditions and variable geometry, especially at stenotic sites, will influence the distribution of the cellular elements in the lumen of blood vessels.[25] Thus, not surprisingly, variable amounts of RBCs can be detected in thrombi removed surgically from large arteries (Supplemental Figure I). The major goal of the present study was to evaluate the contribution of RBCs to clot stability from the aspect of fibrinolysis. Because in a plasma environment RBCs could affect the final clot structure through interactions at multiple steps of the coagulation process (eg, exposing negatively charged phospholipids that provide surface for the assembly of coagulation factor complexes)[26] and to avoid the interference of plasma inhibitors, we evaluated clots prepared from purified fibrinogen and washed RBCs. Thus, initiating dissolution with plasminogen and tPA at precisely known concentrations a relatively limited number of variables needed to be considered in the evaluation of the fibrinolytic side of the hemostatic balance. The study of clot dissolution was approached in 2 experimental setups. In the model with incorporated tPA, the activator was uniformly dispersed within the RBC-fibrin clots at a concentration of 1 nmol/L, which is relevant to the amount of tPA measured in human arterial thrombi,[27] thus mimicking the situation in hemostatic plugs at sites of vascular injury when coagulation and fibrinolysis are initiated simultaneously. In the second setup, tPA was applied to the surface of preformed RBC-fibrin clots at a concentration of 60 nmol/L, relevant to the values maintained in blood in the course of enzymatic thrombolysis,[28] thus mimicking the therapeutic systemic administration of plasminogen activators. Despite the differences in the experimental approaches, in both settings the effect of RBCs was consistent; increasing RBC occupancy of the clot resulted in resistance to lysis (Figures 3 and 5C). This antifibrinolytic effect of RBCs, which is expressed at physiologically relevant RBC counts, shifts the balance of coagulation/fibrinolysis in favor of clotting and may prevent the premature dissolution of hemostatic plugs or contribute to the thrombotic complications in polycythemia vera. In addition, the heterogeneous RBC composition of thrombi (Supplemental Figure I) may be one of the factors contributing to the variability in the lytic susceptibility during thrombolytic therapy of myocardial infarction and stroke.

The mechanism of RBC-related fibrinolytic resistance was approached using ultrastructural and kinetic methods. Scanning electron microscope imaging of the fibrin structures formed in the presence of RBCs evidenced thinner fibers in the RBC-rich areas (Figure 2). These measurements were performed in 2 extreme settings: either keeping the total amount of fibrin constant, with a consequent increase in the extracellular fibrinogen concentration in parallel with the RBC-excluded volume, or at a constant extracellular fibrinogen concentration for any RBC occupancy (Figure 1). In the second setting, a dose-dependent decrease in fiber diameter was observed with increasing RBC occupancy of the clot (Table), whereas in the first setting this effect was counteracted by the thickening effect of rising fibrinogen concentration (Table, reference values), with the outcome seen in Figure 2B and the Table (Model 1, +RBC values). The lysis time measured in the ball sedimentation assay (Figure 3) was inversely correlated with the established changes in fiber diameter and not directly with the RBC occupancy of the clots. When eptifibatide, a blocker of the RBC fibrinogen receptor, reversed the RBC effect on the fiber structure (Figure 2 and the Table), it also reversed the RBC-related inhibition of fibrinolysis (Figures 3 and 5C). Thus, RBC-induced modification of fibrin structure appears to be a major

mechanism in the lytic resistance of clots with RBC content. These results are in agreement with earlier findings that if the fiber diameter is reduced by different factors, the overall rate of fibrin dissolution is slower in similar lytic models with clot-incorporated[19,29] or surface-applied[30] activators.

Because the lytic assays discussed above reflect a global measure of the combined outcome of the 2 stages of fibrinolysis (plasminogen activation and proteolysis of fibrin), separate kinetic and confocal microscopic measurements were performed in attempt to identify discrete effects of RBCs. The plasmin generated in the course of plasminogen activation on the surface of clots in Model 1 increased in the presence of RBCs in the clot (Figure 4A), which is surprising in view of the RBC-related suppression of fibrin dissolution. Furthermore, plasminogen activation on the surface of clots in Model 2 shows a dose-dependent decreasing trend at rising RBC occupancy (Figure 4B). These discordant findings can be explained by the simultaneous action of 2 opposing factors. First, greater RBC occupancy of clots results in thinner fibers (Table), which are a better template for tPA-catalyzed plasminogen activation.[19] Second, the presence of more RBCs in the clot volume results in more cells on the clot surface and a consequent relative increase in the area devoid of plasminogen and inert in terms of plasmin generation. The reduction in interface area occupied by plasminogen-presenting fibrin translates into less plasmin generation. In the setting of the Figure 4A assay, the thinner fiber network gains higher density at increasing fibrinogen concentrations, and thus, the effect of template quality coupled to higher cofactor concentration dominates. In the setting of a constant fibrinogen concentration (Figure 4B), increasing the surface taken by RBCs limits plasminogen activation despite the thinner fibers. Independently of the variations in plasmin generation, in both setups RBCs slow down fibrin dissolution (Figure 3), which suggests that the plasmin-catalyzed proteolytic stage prevails in the control of fibrinolysis at increasing RBC occupancy. The confocal images of fibrin exposed to tPA-GFP (Figure 5A and 5B) provide direct evidence for the limited access of tPA to activatable substrate; the accumulation of tPA in the interfacial fibrin layer is definitely retarded in the presence of RBCs.

This interpretation of the observed changes in plasminogen activation on the surface of RBC-fibrin clots is further supported by the effects of eptifibatide in these assays (Figure 4). This blocker of the RBC fibrinogen receptor increases the fiber diameter (Table) and thus eliminates RBC-related changes in the structure of the fibrin template that favor plasminogen activation,[19] leaving unopposed the effect of the expanding RBC-occupied surface with a consequent decline in plasminogen activation. In the absence of fibrin, eptifibatide stimulates plasminogen activation (data not shown), and this effect persists if activation occurs on the surface of cell-free fibrin with less efficient template function (thicker fibers at 8.2 μmol/L or higher fibrinogen concentration, Figure 4A), but not on the thinner fibers with better cofactor function in the tPA-catalyzed plasminogen activation (Figure 4B). The reported observations for the generation of plasmin in the presence of RBCs indicate that binding of the receptor blocker to RBCs eliminates its direct effect on plasminogen activation and likewise moderates the RBC effects. The data on the effects of eptifibatide in plasminogen activation (Figure 4) and fibrinolytic (Figure 3) assays suggest that the RBC fibrinogen receptor mediates, at least in part, the RBC-related lytic resistance of fibrin.

In summary, our present report unravels a clot-stabilizing function for RBCs based on suppressed tPA-induced fibrinolysis in RBC-modified fibrin structures. In addition to steric hindrance, RBCs modulate fibrinolysis through a specific fibrinogen receptor, as evidenced by the effects of eptifibatide, a cyclic heptapeptide that reversibly inhibits the binding of fibrinogen to $\alpha_{IIb}\beta_3$ integrin. This $\alpha_{IIb}\beta_3$ antagonist is used in clinical practice as an antiplatelet agent,[31] but its newly described role in RBC-containing clots extends our understanding of its favorable antithrombotic action by overcoming the resistance to lysis of RBC-rich thrombi.

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Disclosures

None.

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Supplemental Material

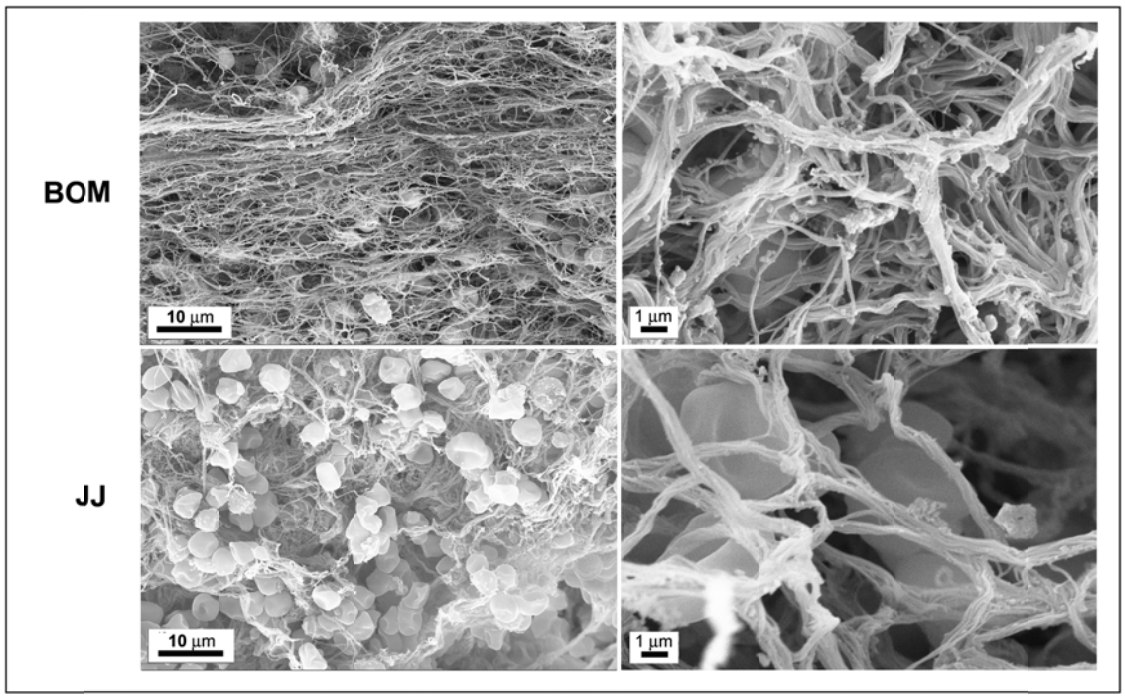

Fig. I Fibrin structure in ex vivo thrombus samples

For illustration of the variable RBC content of thrombi two extreme cases (low and high RBC count) were selected out of 33 thrombi removed with surgery from large arteries. Both samples were from graft vessels (JJ, femoro-popliteal by-pass Dacron graft; BOM, ileo-femoral by-pass homograft from deceased healthy donor). All laboratory findings were in the normal range except for the moderate anemia of BOM (0.29 hematocrit, 9.1 g/dL hemoglobin). No inherited or acquired thrombophilic state was diagnosed in the reported patients. At the time of thrombectomy both patients received heparin treatment. Written informed consent was obtained from all patients and the study protocol was approved by the institutional and regional ethical board. Immediately (within 5 min) after the surgery 5x5x10 mm pieces of thrombi were cut, washed, fixed and dehydrated as detailed in Methods and SEM images were taken. The cellular elements are predominantly RBCs.

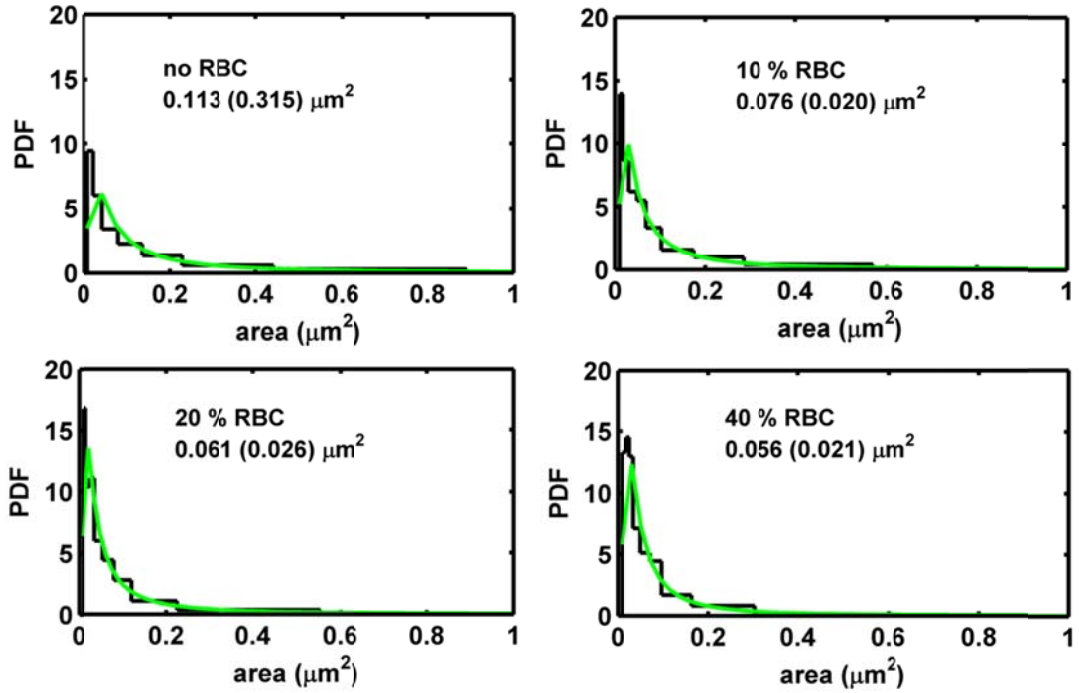

Fig. II Area of pores in the fibrin network

Pore area of the fibrin clots presented in Fig. 2 is evaluated according to algorithm described earlier.[1] The numbers under the RBC content show the median and interquartile range of the fitted distributions for the pore size. The presence of RBC is coupled to smaller pore size (the difference between 10 and 20, as well as 20 and 40 % RBC is not significant, for the rest of the pairs $p<0.05$). A limitation of the approach is related to the fact that when RBC are present, the term pore loses its original meaning in the pure fibrin. RBC fills in the pore, so it's not empty and the evaluation algorithm catches only a fraction of the real pores and not those where RBC occupy the background. Thus, at higher RBC occupancy the distribution is based on a limited number of data, which does not allow reliable estimation of differences between different experimental setups. However, the jump in pore size between cell-free and RBC containing fibrin is large and conclusively supported by the data.

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