HHS Public Access

Author manuscript

J Thromb Haemost. Author manuscript; available in PMC 2020 February 01.

Published in final edited form as:

J Thromb Haemost. 2019 February ; 17(2): 271–282. doi:10.1111/jth.14360.

Red blood cells: the forgotten player in hemostasis and thrombosis

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Summary

New evidence has stirred up a long-standing but undeservedly forgotten interest in the role of erythrocytes, or red blood cells (RBCs), in blood clotting and its disorders. This review summarizes the most recent research that describes the involvement of RBCs in hemostasis and thrombosis. There are both quantitative and qualitative changes in RBCs that affect bleeding and thrombosis, as well as interactions of RBCs with cellular and molecular components of the hemostatic system. The changes in RBCs that affect hemostasis and thrombosis include RBC counts or hematocrit (modulating blood rheology through viscosity) and qualitative changes, such as deformability, aggregation, expression of adhesive proteins and phosphatidylserine, release of extracellular microvesicles, and hemolysis. The pathogenic mechanisms implicated in thrombotic and hemorrhagic risk include variable adherence of RBCs to the vessel wall that depends on the functional state of RBCs and/or endothelium, modulation of platelet reactivity and platelet margination, alterations of fibrin structure and reduced susceptibility to fibrinolysis, modulation of nitric oxide availability, and the levels of von Willebrand factor and factor VIII in blood related to the ABO blood group system. RBCs are involved in platelet-driven contraction of clots and thrombi that results in formation of a tightly packed array of polyhedral erythrocytes, or polyhedrocytes, which comprises a nearly impermeable barrier important for hemostasis and wound healing. The revisited notion on the importance of RBCs is largely based on clinical and experimental associations between RBCs and thrombosis or bleeding, implying that RBCs are a prospective therapeutic target in hemostatic and thrombotic disorders.

Keywords

red blood cells; erythrocytes; hemostasis; thrombosis; blood clotting

Introduction

Studies of erythrocytes, or red blood cells (RBCs), has been a major focus of hematology, as has been hemostasis and thrombosis, but until recently, there has been little overlap in these

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two areas, since most scientists and clinicians have assumed that RBCs play a largely passive and relatively unimportant role in thrombosis and hemostasis. However, now it has become apparent that RBCs have a variety of important functions and exert substantial influences on blood clotting, hemostasis and thrombosis that are clinically significant (Table 1). This notion is based on the major observations that include reduced bleeding at a high hematocrit irrespective of the platelet count and predisposition to thrombosis associated with an increase in the RBC count, congenital erythroid diseases, as well as various acquired pathological conditions that change the properties of RBCs (Table 2). A relatively high incidence of thrombotic complications after RBC transfusions provides another strong argument for the involvement of RBCs in blood clotting disorders, although the thrombosis risk could also be ascribed to the underlying disease, which may dampen the causality of blood transfusion for thrombosis. In addition to clinical observations and experimental studies, computational modeling of thrombosis with a focus on the effects of RBCs has provided quantitative and mechanistic insights [1]. This review will briefly summarize what is currently known about the involvement of RBCs in hemostasis and thrombosis and its underappreciated importance.

Quantitative and qualitative changes in RBCs related to bleeding and thrombosis

Hematocrit and rheological effects

It has long been known that low hematocrits are associated with prolonged bleeding times, even if the platelet counts are normal [2]. Consequently, many bleeding disorders have been corrected by transfusion of RBCs, despite normal or even low platelet levels. Conversely, patients with an abnormally high hematocrit, such as those with polycythemia vera or taking erythropoietin, including doping by healthy athletes [3], are more susceptible to thrombotic disorders [4]. Thus, for some time there has been indirect but solid evidence that RBCs do play some role in hemostasis and thrombosis and can be procoagulant or prothrombotic.

RBCs contribute to blood viscosity, which increases non-linearly with hematocrit and comprises a pathogenic mechanism for thrombosis (Fig. 1). The increased viscosity slows down the flow and can be a strong prothrombotic factor as a component of Virchow’s triad, which accounts for the pathophysiological mechanisms of thrombosis as a combination of endothelial damage, hypercoagulability, and disturbance of blood flow. Such increases in blood viscosity may promote platelet margination and have physical effects on the interaction between platelets and the blood vessel walls, since platelet adhesion increases with hematocrit. Therefore, physical effects of RBCs on hemostasis and thrombosis depend on both the hematocrit and flow conditions [5].

The commonly observed direct correlation between hematocrit and the prothrombotic phenotype has exceptions. Elevated hematocrit in animal models of polycythemia vera or erythropoietin-induced erythrocytosis did not correlate with thrombosis. Moreover, enhanced $FeCl_3$-induced thrombosis in polycythemia vera mice was associated with an increased tail bleeding time, perhaps due to simultaneous deficiency of GPVI and impaired multimerization of von Willebrand factor [6]. The same bleeding tendency was revealed in

mice with extremely high hematocrit (85%), while animals with a lower hematocrit were indistinguishable from controls in a thrombosis model, suggesting that the prothrombotic effects of RBCs may be compensated for with other mechanisms [7]. Therefore, the relationship of the RBC content to thrombosis may be not straightforward and so worth further investigation.

Deformability

Physiologically, RBCs that are 7–8 μm in size must change from their native biconcave shape to a bullet-like shape every time they squeeze through 1–3-μm blood vessels to maintain a high surface area necessary for efficient exchange of oxygen and carbon dioxide between blood and tissues. The efficacy of this diffusive exchange is determined by maximizing the active contact area between a RBC and the vessel wall, due to the deformation of RBCs and a high surface-to-volume ratio. The biconcave discoid shape compared to spherical provides approximately 40 μm^2 (43%) of additional surface area. Deformability of RBCs depends mainly on cytoskeletal proteins and intracellular viscosity [8]. RBCs have a remarkably soft cytoskeleton under the plasma membrane that has a special dynamical molecular structure comprised of non-covalent association of proteins, namely spectrin, actin, ankyrin, Band 3, Band 4.1, and glycophorin C [9]. Structural alterations of transmembrane or cytoskeletal proteins or composition of membrane phospholipids result in rupture of the RBC membrane (hemolysis) or an increase in membrane stiffness. In addition to a decrease in membrane flexibility, increased RBC rigidity can be caused by changes in the viscosity of the cytoplasm due to an increase in hemoglobin concentration or a decrease in hemoglobin solubility [10]. The intracellular content of ATP used by the ion pumps to maintain the RBC volume through water-ion balance content, as well as increased Ca^{2+} concentration, also reduce RBC deformability. Irrespective of the underlying mechanisms, more rigid RBCs are associated with thrombogenic potential, since they can hardly squeeze through the microvasculature and they also enhance platelet margination (Fig. 2).

There are a number of acquired pathological conditions and inherited diseases with reduced RBC deformability, such as autoimmune hemolytic anemia, sickle cell disease, thalassemia, hereditary spherocytosis and xerocytosis. In sickle cell disease patients, the membrane of RBCs is much stiffer than in normal cells [11]. Besides the increased membrane rigidity, the overall stiffness of cells increases dramatically due to intracellular polymerization of mutated hemoglobin S, resulting in formation of sickled RBCs. Increased stiffness of RBC membranes combined with prothrombotic properties of RBCs have been reported also in β-thalassemia, immune hemolytic anemias, hereditary stomatocytosis, coronary heart disease, hypertension, diabetes, deep vein thrombosis (Table 2). RBC membrane viscosity and rigidity have been shown to correlate directly with RBC-derived reactive oxygen species lipid peroxidation [12]; however, the RBC deformability in conditions of oxidative stress is preserved by nitric oxide [13]. During storage, the rigidity of RBCs increase over time, this may be partly responsible for thrombotic complications of RBC transfusions along with other RBC alterations and with respect to thrombotic risk associated with the primary disease.

RBC aggregation

Another example of the significance of locally altered blood rheology is the formation of roleaux (linear arrays of stacked cells) or three-dimensional aggregates with stasis of blood or at low shear rates [14]. These aggregates increase the blood viscosity and hydrodynamic resistance in larger blood vessels with low shear, such as the veins in the lower limbs [15], again confirming Virchow's triad, as these RBC aggregates promote venous thrombosis (Fig. 1). In very small vessels, aggregated RBCs concentrate along the flow axis and enhance platelet margination, and cause a decreased local viscosity and reduced flow resistance (Fahraeus effect) [16].

Two alternative mechanisms of RBC aggregation have been conceived, namely a bridging model and local osmotic gradient model [10]. The former model implies that the intercellular interactions are mediated by plasma proteins, mainly fibrinogen and immunoglobulins. The local osmotic gradient or depletion model attributes aggregation of RBCs to a lower protein concentration near the cell membrane compared with the ambient solution, resulting in an osmotic gradient or local depletion interaction [17]. There is increasing evidence supporting the osmotic gradient mechanism of RBC aggregation.

Phosphatidylserine exposure in RBC membrane

An essential component of blood clotting is a procoagulant cellular or cell-derived phospholipid membrane with exposed negatively charged phosphatidylserine that provides a matrix for assembly of coagulation complexes, namely the intrinsic tenase and prothrombinase (Fig. 1). In normal and quiescent cells, phosphatidylserine is located in the inner leaflet of the plasma membrane to separate this procoagulant phospholipid from plasma coagulation factors. Phosphatidylserine becomes exposed by the protein scramblase that abolishes natural membrane phospholipid asymmetry in response to Ca^{2+}-induced inactivation of translocase and flippase that sustain this asymmetry [18]. The exposure of phosphatidylserine and its role in blood clotting has been mostly studied on platelets, but recent data provide evidence that RBCs can expose phosphatidylserine on their membrane and promote thrombin formation [19]. However, it has been proposed that RBCs may have dual pro- and anticoagulant activity, because they, unlike platelets, generate α-thrombin through an intermediate meizothrombin, a strong protein C activator with low fibrinogen- and PAR-cleaving activity, although the relative significance of the procoagulant effects is greater [19, 20].

RBCs lose membrane asymmetry and expose phosphatidylserine under conditions of cell damage induced by high shear rates, inflammation, or oxidative stress [21]. The exposure and shedding of phosphatidylserine mediated by the intracellular influx of Ca^{2+} is a part of RBC apoptosis and natural cell senescence [22]. The prothrombotic potential of phosphatidylserine exposure in RBCs is large due to the high RBCs count. While ~0.5–0.6% of the RBC population normally expresses phosphatidylserine in healthy subjects and induces some thrombin generation, the contribution of RBCs in pathological conditions may reach 40% of the thrombin-generating potential of whole blood [23].

A substantial amount of phosphatidylserine is exposed on RBC membranes in patients with sickle cell disease and thalassemia [24]. In sickle cell disease, this exposure results from the repeated cell deformation into sickled shapes and back to the biconcave shape due to reversible polymerization of sickle hemoglobin. Interestingly, in the blood of sickle cell disease patients, thrombin generation has been shown to correlate inversely to RBC phosphatidylserine exposure [25], implying that in sickle cell disease, additional procoagulant mechanisms exist unrelated to phosphatidylserine that are described elsewhere [26]. In β-thalassemia, increased phosphatidylserine exposure on the surface of RBCs is associated with the cell death pathway named eryptosis [27].

RBC-derived microvesicles

Many cells, including RBCs, generate microscopic extracellular membranous structures called microvesicles (MVs) or microparticles as a result of activation, apoptosis or aging. Membrane blebbing and formation of MVs is a consequence of the loss of membrane phospholipid asymmetry on RBCs, which is why MVs bare phosphatidylserine on their surface [28]. MV generation in RBCs results from a disturbance of the membrane-cytoskeleton interactions [29]. While MVs were once thought to be an undesirable byproduct of these processes, it is now known that they represent a means for intercellular communications *in vivo*, an important regulatory mechanism of physiologic reactions, and a pathogenic component in many thrombotic and hemostatic disorders (Fig. 2) [30]. Furthermore, MVs from RBCs accumulate during storage of whole blood [31] which might be partially responsible for an increased incidence of deep vein thrombosis or other thrombotic conditions after transfusion of RBCs stored for longer times [32]. MVs from RBCs increase in sickle cell disease and hemolytic anemia, and other prothrombotic states associated with RBCs [33]. Higher levels of MVs in the plasma are associated with a dose- and time-dependent increase in generation of thrombin and a reduction in clotting time, suggesting that they enhance hypercoagulability [34]. This enhanced thrombin generation has been associated with expression of phosphatidylserine [28]. Alternatively, RBC-derived MVs can initiate thrombin formation via a factor XII-dependent pathway without tissue factor activity [35]. The circulating MVs can also promote vaso-occlusion by internalizing free heme and transferring it to vascular endothelium, or activate the complement system [36]. Altogether, RBC-derived MVs, either formed *in vivo* or infused along with stored RBCs, have prothrombotic effects with multiple underlying mechanisms [37, 38]. With all of these procoagulant activities, RBC-derived MVs could be a target for treatment of thrombotic disorders [39].

RBC storage

During storage for transfusions, RBC preparations develop multiple and diverse changes in their structure and metabolism caused by the accumulation of their own waste products, by enzymatic and oxidative injury, and by programmed cell death [40]. These alterations are altogether designated as “storage lesion” and include a decrease in the content of 2,3-diphosphoglycerate and ATP concentrations, membrane loss, shape changes, formation of MVs, and release of toxic products, such as extracellular hemoglobin associated with hemolysis, lysophospholipids, and iron ions [41]. Storage of RBCs is accompanied by strong procoagulant changes, such as exposure of phosphatidylserine on cells as well as on the

abundant RBC-derived MVs (Fig. 2) [40]. High concentrations of procoagulant phosphatidylserine-expressing MVs formed in preparation of stored RBCs exaggerate thrombotic complications after RBC transfusion [42]. In addition, free extracellular hemoglobin resulting from storage-related hemolysis binds and inactivates nitric oxide in blood, a potent vasodilator and inhibitor of platelet activation, which is another prothrombotic consequence of RBC infusions [43]. Therefore, infusions of RBCs, especially old ones, have adverse thrombotic effects, among which deep vein thrombosis is one of the most common [32]. In particular, perioperative RBC transfusions have been shown to be associated with a higher incidence of postoperative venous thromboembolism on top of the risk associated with surgery itself [44]. In addition to transfusions of normal stored RBCs, chemically modified and loaded RBCs have been studied intensively as cargo for targeted drug delivery [45].

Hemolysis

Hereditary and acquired hemolytic anemias, of which immune hemolysis is the most common, arise from hemolysis, with release of free, extracellular hemoglobin into the blood (Fig. 2). Massive hemolysis followed by thrombotic complications is a major pathogenic mechanism in paroxysmal nocturnal hemoglobinuria [46] and of adverse effects of RBC transfusions, provided they are not caused by the underlying disease [47, 48]. Hemolysis is accompanied by (pro)thrombotic conditions that can range from mild hypercoagulability detected by laboratory signs to life-threatening complications, such as disseminated intravascular coagulation and venous thromboembolism [49]. Hemolysis may result in such prothrombotic conditions via several pathogenic mechanisms. Hemolysis is commonly accompanied by a large release of RBC-derived MVs, with all of the effects described above [35]. Free hemoglobin and heme, which are toxic to many cells and tissues, are released [50]. Furthermore, extracellular hemoglobin sequesters NO and thus enhances adhesion/aggregation of platelets and activation of endothelial cells [51]. Free heme also generates reactive oxygen species, upregulates heme oxygenase activity, and directly activates macrophages and endothelial cells [52]. Finally, immune hemolysis is accompanied by activation of the complement cascade and production of TNF-α, which induces tissue factor expression in endothelial cells and decreases the endothelial expression of thrombomodulin, down-regulating the anti-coagulant pathway [49].

Although extracellular hemoglobin release during hemolysis has been considered as a transporter and scavenger of nitric oxide (NO), an inhibitor of endothelial cells and platelets, and a vasodilator, it has been shown recently that hemoglobin can preserve functional effects of NO by formation of S-nitrosothiols bound reversibly to the Cys-93 residue of the β-chain [53]. S-nitrosothiols have antiplatelet activity similar to NO and therefore lysed RBCs can either enhance platelet aggregation by scavenging NO or inhibiting platelets through release of functional equivalents of NO [48].

Interactions of RBCs with cellular and molecular components of the hemostatic system

Vessel wall

The interaction of RBCs with endothelium under physiological conditions is minimal, but they become adhesive when RBCs and/or endothelial cells undergo pathological perturbations, resulting in occlusion of the microvasculature, often associated with thrombotic conditions. Increased RBC adhesion to endothelium is mediated by a number of adhesive molecules, such VCAM-1, α4β1, Lu/BCAM, ICAM-4, etc. [10, 54]. In addition to the interaction of RBCs with activated endothelial cells, they can be exposed and bind to subendothelial matrix when the endothelium is damaged. The RBC-endothelium adhesive interaction causing occlusion of small vessels has been shown in a number of pathological conditions, such as retinal venous occlusion, hypertension, diabetic mellitus, and stroke (Table 2). RBC adhesion to endothelium in central retinal vein occlusion is mediated by the interaction between phosphatidylserine exposed on the surface of RBCs and endothelial phosphatidylserine receptor [55]. Prolonged storage followed by time-dependent alterations of RBCs enhance the ability of infused RBCs to bind to the endothelium and form micro-aggregates that impair blood flow in the microvasculature [56]. In one of the most commonly used vascular injury models in mice for thrombosis using $FeCl_3$, RBCs were shown to be the first cells to adhere to the chemically injured endothelium, but this interaction is an artifact of the $FeCl_3$ [57]. It has been shown recently that dysfunction or loss of CD59, a protective glycoprotein that prevents formation of the complement-dependent membrane attack complex, is a major arterial prothrombotic factor in paroxysmal nocturnal hemoglobinuria. To alleviate consequences of massive hemolysis under this pathological condition, CD59 reduces endothelial damage and platelet activation, as well as their aggregation with leukocytes that exaggerate vascular occlusion [58].

Platelets

A purely rheological effect of RBCs is that they preferentially move down the center of blood vessels, causing margination of platelets, so that they are adjacent to the vessel wall, where they can interact to form a temporary plug in case of injury [59]. This peripheral layer also contains plasma with clotting factors and neutrophils, important for hemostasis. As a result of the RBCs being in the center of the channel and plasma at the periphery, there is a decrease in viscosity at lower vessel diameters, except in capillaries that are smaller than RBCs, where the viscosity of the RBC-free layer increases because of the presence of platelets, which have a greater viscosity than RBCs [60]. One consequence of an elevated hematocrit is increased margination of platelets, enhancing their interactions with the endothelium, perhaps accounting for increased thrombotic complications. Another consequence of the reduced viscosity near the vessel wall and decreased wall shear stress is a reduction in NO release [61]. Since NO prevents activation of endothelial cells and platelets, this NO deficiency results in increased cellular activation.

RBCs can interact directly with platelets at venous shear rates [62], which may be important in prothrombotic pathological conditions, such as thalassemia [63] or sickle cell disease [64]. The ability of RBCs to directly bind activated platelets may play some role in the

unexpected prevalence of RBCs in arterial thrombi that have been traditionally called “white” thrombi made mainly of activated platelets and fibrin [65].

RBCs can also modulate platelet reactivity directly through chemical signaling [66]. Under low oxygen pressure, low pH, and in response to mechanical deformation, RBCs release ATP and ADP, which activate platelets [67]. Release of extracellular hemoglobin from damage to RBCs enhances platelet activation by lowering NO bioavailability [68], since the hemoglobin is a strong NO scavenger, preventing the suppressive effect of nitric oxide on platelet activation [69]. An additional effect is the release from damaged RBCs of arginase, which cleaves L-arginine, a substrate for NO production [68].

Fibrinogen and fibrin

Hyperfibrinogenemia is associated with aggregation of RBCs in a form of roleaux, a morphological sign of (pro)thrombotic conditions and a pathogenic mechanism of microthrombosis [70]. The association between the fibrinogen plasma concentration and the erythrocyte tendency to aggregate was reported in metabolic, inflammatory and vascular diseases [71]. Formation of the aggregates of RBCs occurs through fibrinogen that bridges adjacent cells, similar to the role of fibrinogen in platelet aggregation. It has been shown that binding of fibrinogen to the RBC membrane may be mediated by an integrin-like receptor [72, 73] or an integrin-associated protein (CD47) [74]. The involvement of a β3 integrin of RBCs is supported by the observation that in a patient with mutated αIIbβ3 (Glanzmann’s thrombasthenia) interaction of RBCs with fibrinogen is impaired [73]. Remarkably, the interactions of fibrinogen with RBCs circulating in the blood for a longer time are gradually reduced, perhaps due to desialization of membrane proteins in older RBCs [75]. A recent paper showed that the minor heterozygous fibrinogen variant containing two splicing variants of the γ chain called γA and γ′ bound stronger to RBCs that the major homozygous fibrinogen fraction γAγA [76]. Because fibrinogen and fibrin share binding sites to the integrin αIIbβ3 [77], the specificity of fibrinogen binding to RBCs can be similar to RBC-fibrin interactions in blood clots and thrombi. In addition, retention of RBCs in venous thrombi at a low flow speed and stasis can be mediated by their interaction with von Willebrand factor located either on fibrin on another surface [78]. Understanding the molecular nature of the RBC binding to fibrinogen and fibrin is important because prevention and/or disruption of this interaction may be a novel antithrombotic therapeutic target similar to platelet-fibrin(ogen) interactions.

Clot structure and fibrinolysis

RBCs are incorporated into all types of clots and thrombi formed *in vivo*, especially in the venous system [79] but even in arterial thrombi [65]. Thus, the effects of RBCs on clot structure have been studied *in vitro*. Intermediate RBC concentrations cause considerable heterogeneity in the fiber network, with pockets of densely packed fibers alongside regions where fibers are sparse [80]. With higher levels of RBCs, fibers are more uniformly but loosely arranged around the cells, and fiber diameters are larger. RBCs embedded into a blood clot exclude fibrin and platelets and thereby enlarge the pores of the fibrin network making it more permeable if they are washed out, but at same time they can make the entire clot less permeable because those RBCs that remain (especially those compressed during

contraction) make a physical barrier or a seal that hampers diffusion or perfusion. The modulation in clot structure and mechanical properties from RBCs affects clot stability, embolization, and the efficacy of anticoagulation and therapeutic thrombolysis [80]. In a venous model, RBC retention within clots determines thrombus size dependent on factor XIIIa activity [81, 82], via crosslinking of the fibrin α chains [83].

All of these effects of RBCs on the physical and chemical properties of clots have striking effects on clot dissolution via fibrinolysis. Overall, incorporation of RBCs increases the resistance to lysis and decreases the permeability of clots in a dose-dependent manner [84]. As expected from *in vitro* studies demonstrating an increase in mechanical stability and retardation of fibrinolysis, similar effects were shown for thrombi in experimental cerebral ischemia [85]. Alternatively, RBC-derived MVs have a prominent fibrinolytic activity *in vitro* due to the presence of plasminogen on their surface [86]. Moreover, a higher fraction of RBCs in cerebral thrombi has been shown to correlate with better responsiveness to intravenous thrombolysis [87]. Collectively, the effects of RBCs on fibrinolysis are controversial and need further attention.

Clot contraction

Clot contraction, also known as retraction, is the volume shrinkage of the blood clot that occurs when activated platelets pull on the fibrin network. Non-muscle myosin IIa inside the platelet interacts with actin filaments attached to the membrane-associated adhesive integrin αIIbβ3 via talin and kindlin. Fibrin or fibrinogen bind to αIIbβ3 outside the platelet to link other platelets into a platelet-fibrin meshwork that undergoes mechanical compaction, causing compression of RBCs embedded into the clot [88]. Clot contraction has a number of potential pathophysiological implications. First, it is biologically important that compaction of RBCs reduces the space between cells, due to more efficient packing, which helps to create an impermeable seal at the site of vessel injury to prevent bleeding [89]. Second, contraction pulls clots or thrombi closer to the vessel wall so that they are less obstructive. Third, contraction can modulate effects of the fibrinolytic enzymes by changing clot permeability and spatial proximity of the fibrin fibers. Fourth, clot contraction pulls together wound edges making this process important at the early stages of wound healing.

The kinetics and the extent of clot contraction depend on variations in the molecular and cellular blood composition, including fibrinogen concentration, RBC and platelet count [90]. Unlike thrombin activity, factor XIIIa-catalyzed crosslinking of fibrin, and platelet count, which all enhance the rate and efficacy of clot contraction, RBCs delay and reduce the process of clot compaction, while increasing the mechanical force generated by the activated platelets due to viscoelastic properties and mechanical resilience of RBCs [82, 83, 90]. The examination of how the presence of RBCs affects the compaction of a thrombus, and consequently the blood flow past the thrombus, has the potential to guide future therapeutic applications and has clinical implications in patients with pathological conditions associated with increased (polycythemia) or reduced (anemia, hemodilution) RBC count in the blood.

Platelet contraction propagated via the fibrin network results in the compaction of erythrocytes to the core of the clots and redistribution of platelets and fibrin toward the outside of the clot [89]. The erythrocytes amassed in the core of the contracting clot undergo

a shape transformation from their native biconcave shape to that of polyhedral (Fig. 3), hence named polyhedrocytes [89]. This remarkable polyhedral shape of erythrocytes is a natural morphological form of erythrocytes in addition to echinocytes, acanthocytes, spheroechinocytes, ovalocytes, elliptocytes, stomatocytes, and more. Polyhedrocytes have been observed in *ex vivo* clots and thrombi obtained from human and murine samples and can be used a morphological sign of intravital contraction of clots, thrombi, and thrombotic emboli. In particular, polyhedrocytes have been observed in human arterial and especially venous thrombi, and pulmonary emboli, taken from patients [89, 91–93]. Unexpectedly, the rate and extent of clot contraction has been found to be impaired in a number of (pro)thrombotic conditions, such as ischemic stroke [94], deep vein thrombosis [95], and systemic lupus erythematosus [96], and may be considered an underappreciated factor that increases the risk and/or exaggerates the course and outcome of thrombosis. Altogether these data suggest that the extent of clot compaction and the formation of compressed RBCs (polyhedrocytes) comprise an important pathogenic mechanism of thrombosis and could have a diagnostic and/or predictive value in (pro)thrombotic states.

Factor VIII and von Willebrand factor

An understudied and underappreciated mechanism of the effects of RBCs on bleeding and thrombosis is modulation of the levels of von Willebrand factor and factor VIII in blood, which is related to the ABO blood group system [97]. In subjects that have A and B antigens, the concentrations of factor VIII and, especially, von Willebrand factor are substantially higher than in the O group individuals. This difference has been attributed to the posttranslational glycosylation of the proteins stimulated by the A and B antigens via an unknown mechanism [98]. Presumably, the extent of glycosylation could determine the clearance rate of factor VIII and von Willebrand factor, which results in faster elimination in O blood type subjects than in non-O individuals [99]. The higher levels of von Willebrand factor (and to a lesser extent of factor VIII) might underlie the association between the ABO blood group and the risk of thrombosis or bleeding [100].

Conclusions

There are both quantitative and qualitative changes in RBCs that affect bleeding and thrombosis, as well as interactions of RBCs with cellular and molecular components of the hemostatic system. Low hematocrits are associated with bleeding while high hematocrits are associated with thrombosis, as is the formation of RBC aggregates. Both the stiffness of RBCs and the exposure of phosphatidylserine to form a procoagulant surface that enhances thrombin generation can contribute to thrombosis. MVs from stored RBCs or from some pathological conditions have strong procoagulant effects, as do extracellular hemoglobin and heme as a result of hemolysis. Rheological effects of RBCs include their being concentrated in the center of blood vessels with consequent platelet margination, including effects on viscosity. RBCs interact with endothelial cells and platelets, both of which may be significant for thrombosis. RBCs also interact with fibrin(ogen) and affect the structure, mechanical properties, and hence the lytic resistance of clots and thrombi. Clot contraction may be important for hemostasis and wound healing because contracted clots form an impermeable barrier made of tightly packed polyhedrocytes. Furthermore, clot contraction

restores blood flow past otherwise obstructive thrombi, though in some prothrombotic conditions platelet activation and exhaustion leads to a lower extent of clot contraction. In summary, the ability of RBCs to affect hemostasis and thrombosis is multifactorial and has multiple underlying mechanisms: modulating blood viscosity via hematocrit, deformability, and aggregation; variable adherence to the vessel wall that depends on the functional state of RBCs and/or endothelium; modulation of platelet reactivity; platelet margination; release of MVs; membrane composition (expression of adhesive proteins and phosphatidylserine); modulation of nitric oxide availability; expression of blood group antigens implicated in thrombotic and hemorrhagic risk. Most of these effects (summarized in Figs. 1 and 2) are prothrombotic and result in promotion of arterial and venous thrombosis. However, the RBC-related influences are much more complex and may be either pro- or antithrombotic (Table 1), making analysis of the biological role of RBCs not straightforward and emphasizing the need for further investigation.

Acknowledgments

Disclosure of Conflict of Interest

J.W. Weisel's work was supported by NIH grants UO1HL116330, HL135254, NSF grant DMR1505662, and a grant from Boston Scientific. R.I. Litvinov has received support from the Program for Competitive Growth at Kazan Federal University.

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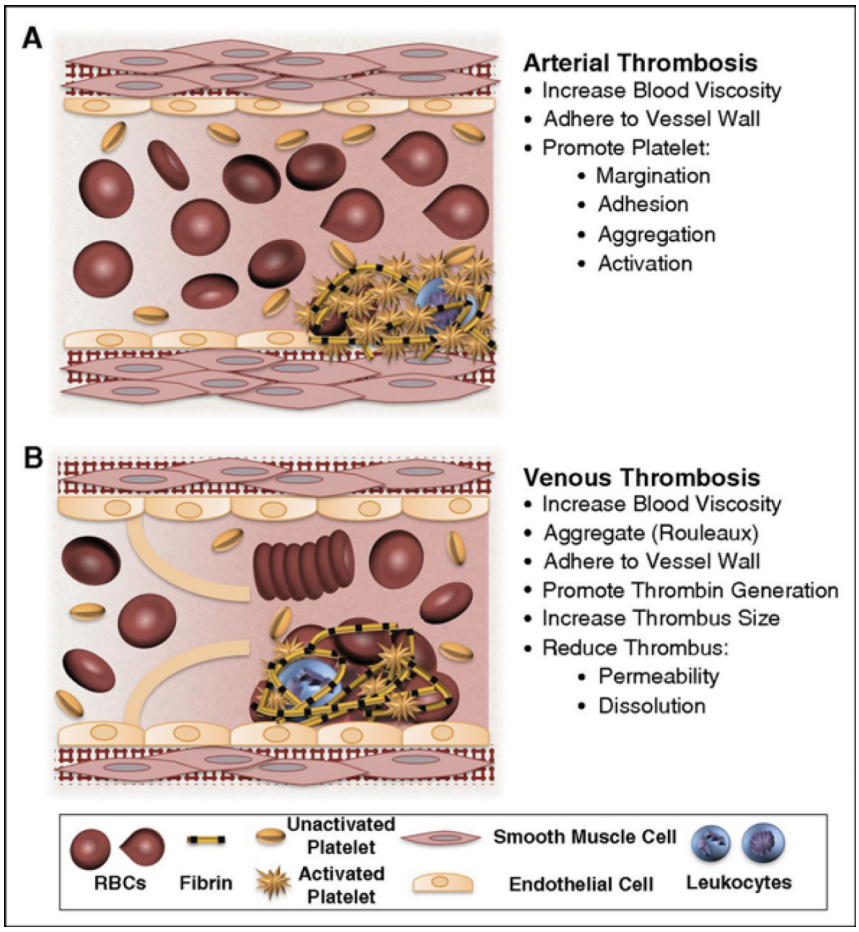

Figure 1.

Potential contributions of normal and abnormal RBCs to arterial and venous thrombosis/thromboembolism. (**A**) Arterial thrombi arise in vessels with high shear rates, which promotes the rapid formation of platelet-rich thrombi. During arterial thrombosis, RBCs promote platelet margination, increase platelet-thrombus interactions, and enhance platelet adhesion and activation. Although RBCs increase blood viscosity, this effect is lessened in arteries by high shear-induced shape change. (**B**) Venous thrombi form slowly in stasis or low flow (frequently in venous valve pockets) and are RBC and fibrin rich. In veins, RBC aggregation into stacked rouleaux structures increases blood viscosity. RBCs can also directly or indirectly adhere to the vessel wall and may contribute to thrombin generation within thrombi. Once incorporated into venous thrombi, RBCs increase thrombus size and reduce thrombus permeability and susceptibility to lysis. In disease states, abnormal RBCs and RBC-derived microvesicles may also adhere to the endothelium or extracellular matrix, activate platelets and other cells, and enhance local thrombin generation during thrombosis. [With permission from: Byrnes JR, Wolberg AS. Red blood cells in thrombosis. Blood 2017, **130**: 1795]

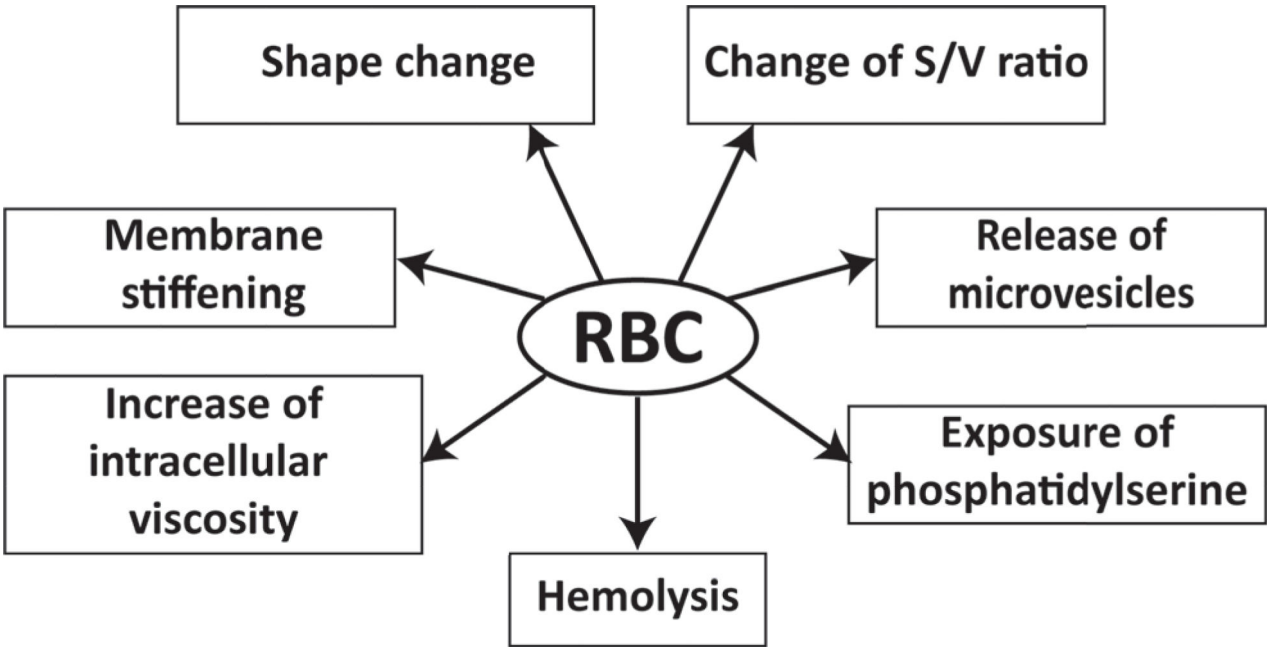

Figure 2.
Pro-thrombotic alteration of RBCs in various disease states and during storage. S/V = surface to volume ratio.

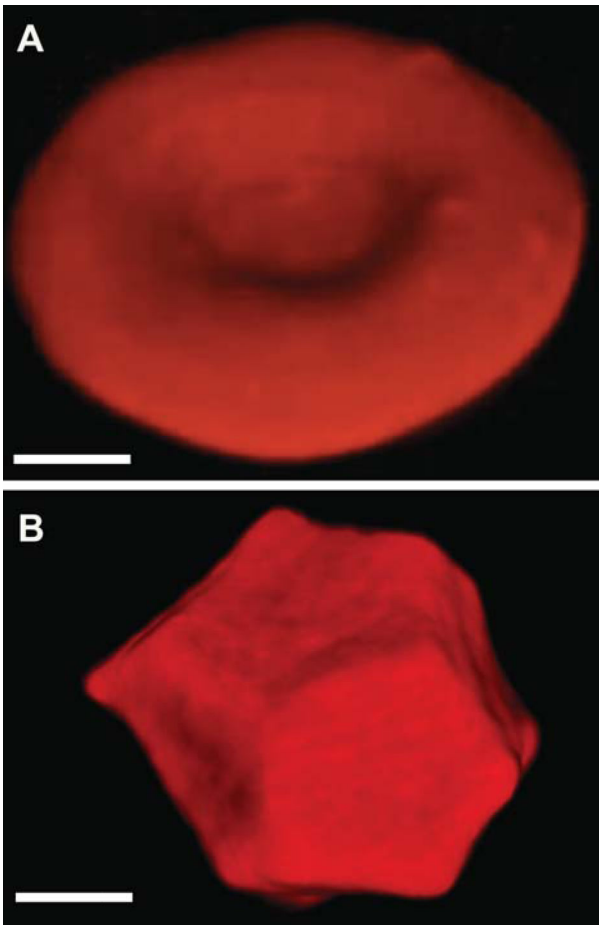

Figure 3.

Three-dimensional confocal microscopy images of a native biconcave RBC (**A**) and a compressed multi-faceted polyhedral RBC, or polyhedrocyte (**B**), formed as a result of blood clot contraction. Magnification bars = 1 μm.

Table 1.

Effects of RBCs related to thrombosis and hemostasis and underlying mechanisms

Effects	Mechanisms	Pro- or antithrombotic	References ##
Hemorheological effects	RBCs increase blood viscosity because of a rise in hematocrit, an increase in RBC aggregation, or a decrease in RBC deformability (increasing flow resistance)	Pro	2, 3, 4, 5
	Conversely, anemia is associated with low blood viscosity and bleeding tendency due to reduced platelet margination toward endothelium and enhanced NO availability	Anti	2, 3, 4, 5
	RBCs undergo shear-dependent reversible aggregation mediated by plasma proteins (mainly fibrinogen, immunoglobulins) and/or local osmotic gradient	Pro	14, 15, 16, 70, 71, 72, 73, 74
	RBCs with increased rigidity occlude small vessels	Pro	11, 12
	Deformability of RBCs reduces frictional resistance to flow	Anti	8, 11, 12, 13
	RBC maintain biconcave shape and a high surface-to-volume ratio due to cytoskeleton and water/ions balance	Pro or Anti	5
	RBCs migrate to the center of blood flow and push platelets toward the endothelium (margination) in a hematocrit- and shear-dependent manner	Pro	59, 60, 61
Effects on platelet reactivity	RBCs increase platelet adhesion and aggregation by release of ADP and thromboxane A_2	Pro	66, 67
	RBC form aggregates with platelets via adhesive molecules (ICAM-4 and fibrinogen with αIIbβ3)	Pro	62, 63, 64
	Free hemoglobin released during hemolysis scavenges nitric oxide, a platelet inhibitor and vasodilator	Pro	50, 51, 68, 69
	Free hemoglobin suppresses platelet activation by release of S-nitrosothiols, functional equivalents of NO	Anti	48, 53
Interactions with vessel wall	RBCs bind directly to endothelium via adhesive molecules (Lutheran blood group/basal cell adhesion molecule/band 3, integrin $\alpha_{4B}\beta_1$, CD36, ICAM-4, phosphatidylserine, etc.)	Pro	10, 54, 55
	In $FeCl_3$-induced thrombosis RBCs bind to endothelium via unknown mechanisms	Pro	57
	RBCs modulate endotheliocyte activation through release of NO, NO equivalents, and ATP	Anti	49, 52
Thrombin generation	Phosphatidylserine is exposed on RBCs by Ca^{2+}-dependent scramblase in response to high-shear stress, complement attack, oxidative stress, apoptosis, etc.	Pro	18, 19, 20, 21, 22, 23, 24, 25
	RBCs release membrane-derived procoagulant microvesicles bearing phosphatidylserine during *in vivo* aging and *in vitro* storage	Pro	28, 29, 30, 31, 33, 34
	Meizothrombin, a protein C activator with low fibrinogen-cleaving activity, is formed on RBCs and released into the blood	Anti	20
	Factor IX is activated directly by an elastase-like enzyme on the RBC membrane	Pro	
Structure and properties of clot and thrombi	RBCs make the fibrin network more porous	Anti	65, 79, 80
	Variable deformability of RBCs affect blood clot mechanics	Pro or Anti	
	Factor XIIIa-mediated RBC retention increases thrombus size	Pro	81, 82, 83

Effects	Mechanisms	Pro- or antithrombotic	References ##
Effects on fibrinolysis and thrombolysis	RBCs reduce clot permeability	Pro	84, 86, 87
	RBCs suppress tPA-induced plasminogen activation	Pro	
	RBCs decrease fibrin fiber dimeter and change the network structure, thus reducing susceptibility to fibrinolysis	Pro	
	RBCs are potential transportation cargo for targeted delivery of thrombolytic drugs	Anti	45
Effects on clot contraction	Compacted RBCs form impermeable seal	Pro or Anti	88, 89
	RBCs undergo compressive deformation from biconcave to polyhedral and intermediate forms	Pro or Anti	91, 92, 93
	RBCs are redistributed in contracted clots toward the middle	Pro or Anti	89
Hemostatic effects of RBC transfusions	RBC transfusion stops bleeding associated with anemia and thrombocytopenia	Pro	39, 47
	RBC transfusion improves platelet responsiveness to stimulation	Pro	
Complications of RBC transfusions	“Storage lesion” of RBCs includes: - oxidative stress and membrane damage - phosphatidylserine exposure - release of microvesicles - hemolysis - increased membrane rigidity - release of free hemoglobin - activation of complement - depletion of NO and its functional equivalents - apoptosis (eryptosis)	Pro	31, 32, 34, 36, 37, 40, 41, 42, 43, 44, 45, 47, 48, 49

Table 2.

(Pro)thrombotic pathologies with RBCs as a (major) pathogenic factor

Pathologies	References
Erythroid diseases	
Polycythemia vera	*J Intern Med* 1998, **244**: 49; *Curr Opin Hematol* 2014, **21**: 186
Hereditary elliptocytosis	*Int J Lab Hematol* 2017; **39** Suppl 1: 47
Hereditary stomatocytosis	*Br J Haematol* 1996, **93**: 303; *Blood* 1997, **89**: 3451
Hereditary spherocytosis	*Blood* 2009; **114**: 2861; *J Thromb Haemos*t 2008; **6**: 1289; *Curr Opin Hematol* 2014, **21**: 186
Hereditary xerocytosis	*Int J Lab Hematol* 2017; **39** Suppl 1: 47; *Rev Med Interne* 2007; **28**: 879
(Beta)-thalassemia	*Acta Hematol* 1992, **87**: 71; *Stroke* 1990, **21**: 812; *Am J Physiol* 1996, **270**: H1951
Sickle cell disease	*Thromb Haemost* 1996, **76**: 322; *Curr Opin Hematol* 1996, **3**: 118; *Microcirculation* 2009, **16**: 97
Paroxysmal nocturnal haemoglobinuria	*Blood Cells Mol Dis* 2017, **65**: 29; *Br J Haematol* 2011; **152**: 631
Glucose-6-phosphate dehydrogenase deficiency (favism)	*Vox Sang* 2013; **105**: 271
Secondary erythrocytosis	*Sleep Breath* 2010; **14**: 193
Non-erythroid diseases	
Immune hemolytic anemias	*Br J Haematol* 2016, **172**: 144
Atherosclerotic vascular disease	*Coron Artery Dis* 1998, **9**: 113; *Clin Hemorheol Microcirc* 2004, **31**: 185
Cerebral infarction	*Lancet* 1981, **2**: 114
Coronary heart disease	*Blood* 1997, **89**: 4236
Myocardial infarction	*Clin Hemorheol Microcirc* 1999, **20**: 111
Complications of RBC transfusion	*Thromb Res* 2015, **136**: 1204
Retinal venous occlusions	*Am J Ophthalmol* 1983, **96**: 399; *Br J Haematol* 1990, **75**: 127; *Curr Opin Hematol* 2014, **21**: 186
Hypertension	*J Hypertens* 1992, **10**: S69; *Clin Hemorheol Microcirc* 1997, **17**: 193
Diabetes mellitus	*J Biomed Eng* 1993, **15**: 155; *Rom J Intern Med* 2004, **42**: 407; *Biorheology* 2009, **46**: 63
Leg vein thrombosis	*Br J Haematol* 1994, **88**: 174
Stroke	*Curr Opin Neurol Neurosurg* 1992, **5**: 44
Malaria	*Science* 1994, **264**: 1878; *Cell Microbiol* 2013, **15**: 1976
Acquired dysfibrinogenemia	*J Vasc Surg* 1997, **26**: 1061
Systemic inflammation (hypergammaglobulinemia)	*BBA Clin* 2016, **5**, 186; *Cell Death Dis* 2012, **3**: 410
Bacterial sepsis	*J Mol Med* 2007, **85**: 269; *Am J Respir Crit Care Med* 1998, **157**: 421
Gaucher disease	*Br J Haematol* 2006, **134**: 432; *Curr Opin Hematol* 2014, **21**: 186