ISBT Science Series
An affiliated publication to Vox Sanguinis
ISBT International Society of Blood Transfusion
ISBT Science Series (2017)

CONGRESS REVIEW 3C-S12-02

Role of red blood cells in haemostasis and thrombosis

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In contrast to an obsolete notion that erythrocytes, or red blood cells (RBCs), play a passive and minor role in haemostasis and thrombosis, over the past decades there has been increasing evidence that RBCs have biologically and clinically important functions in blood clotting and its disorders. This review summarizes the main mechanisms that underlie the involvement of RBCs in haemostasis and thrombosis *in vivo*, such as rheological effects on blood viscosity and platelet margination, aggregation and deformability of RBCs; direct adhesion and indirect biochemical interactions with endothelial cells and platelets. The ability of stored and pathologically altered RBCs to generate thrombin through exposure of phosphatidylserine has been emphasized. The procoagulant and prothrombotic potential of RBC-derived microparticles transfused with stored RBCs or formed in various pathological conditions associated with haemolysis has been described along with prothrombotic effects of free haemoglobin and haem. Binding of fibrinogen or fibrin to RBCs may influence their effects on fibrin network structure, clot mechanical properties and fibrinolytic resistance. Recent data on platelet-driven clot contraction show that RBCs compressed by platelets pulling on fibrin form a tightly packed array of polyhedral erythrocytes, or polyhedrocytes, which comprises a nearly impermeable barrier important for haemostasis and wound healing. RBCs may perform dual roles, both helping to stem bleeding but at the same time contributing to thrombosis in a variety of ways.

Key words: coagulation, hemostasis, red cell components, red cells, rheology, thrombosis

Introduction

Until recently, little attention has been paid to the potential involvement of erythrocytes, or red blood cells (RBCs), in haemostasis and thrombosis. Moreover, most scientists and clinicians have assumed that they play a largely passive and relatively unimportant role. However, in the past few decades, there has been increasing evidence that RBCs have a variety of active functions in haemostasis and thrombosis that are significant and need to be taken into account in assessing health and disease. This review will summarize the main mechanisms that underlie the involvement of RBCs in blood coagulation and its disorders, including the effects of stored and transfused RBCs.

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Indirect evidence for the influence of RBCs on haemostasis and thrombosis

More than a hundred years ago, it was reported that in anaemia patients, the bleeding time was prolonged irrespective of the platelet count [1]. Fifty years later, a more general correlation between low haematocrit and prolonged bleeding times was found, including correction of the haemostatic parameters by blood transfusion [2]. It was also established that many bleeding disorders can be treated by an increase in RBC count despite low or normal platelet levels [3]. On the other hand, an abnormally high haematocrit is associated with thrombosis, and patients with polycythemia vera or taking erythropoietin are more susceptible to thrombosis and thromboembolism

[4, 5]. These and other observations have provided indirect but strong evidence that RBCs are important players in haemostasis and thrombosis and can act as a procoagulant and prothrombotic blood component.

Rheological effect of RBCs

High haematocrit results in an increase in blood viscosity that impedes the blood flow [6, 7]. These haemorheological effects of RBCs can be a strong prothrombotic factor since the impaired blood flow is a component of Virchow's triad that explains the pathophysiological mechanisms of thrombosis by a combination of hypercoagulability, disturbance of blood flow and endothelial damage [8]. The haematocrit-related blood viscosity may have physical effects on the interaction between platelets and blood vessel surfaces. Under flow conditions, platelet adhesion increases greatly with haematocrit. Thus, the volume fraction of red cells may have a significant impact on haemostasis and thrombosis, with the nature of the effect related to the flow conditions [9].

A remarkable rheological effect of RBCs that affects platelets in haemostasis and thrombosis is that RBCs preferentially move down the centre of blood vessel, causing margination of platelets, so that they are poised to adhere preferentially to the site of vessel wall injury [10]. In addition to platelets, the peripheral layer formed by the axial accumulation of RBCs contains plasma (with clotting factors) and neutrophils. Formation of the RBC-free layer next to the endothelial lining changes local viscosity, such that the viscosity gets smaller with decreasing vessel diameter (known as the Fahraeus–Lindqvist effect), down to 5–7 μm. In capillaries that are smaller than RBCs, the viscosity of the RBC-free layer increases due to the presence of platelets that have a greater viscosity than RBCs [11]. In the presence of RBCs, the distribution of platelets is changed by a few orders of magnitude compared to uniform Brownian diffusivity, resulting in a 3–8x platelet accumulation near the vessel wall [12, 13]. An elevated haematocrit predisposes one to platelet interactions with the activated endothelium, thus promoting haemostasis or thrombosis [14]. Another consequence of the axial RBC accumulation followed by reduction in local viscosity is a decrease in the wall shear stress causing a lesser local release of nitric oxide [15]. Because nitric oxide is known to prevent activation of endothelial cells and platelets, this nitric oxide deficiency promotes cellular activation.

RBC aggregation

At low shear rates or with stasis of blood, RBCs tend to form linear arrays of stacked cells (rouleaux) or three-dimensional aggregates [16]. Such aggregates are difficult to disperse, and they tend to increase the blood viscosity and hydrodynamic resistance in larger blood vessels with low shear, such as the veins in the lower limbs. RBC aggregation promotes thrombosis in veins, confirming the pathogenic importance of locally altered blood rheology in the development of venous thrombosis [17].

RBC deformability

RBCs are remarkably deformable, which is important to minimize their resistance to flow and to allow them to fit through blood vessels smaller than their size. The great deformability of RBCs is primarily a consequence of their biconcave shape, specifically the high surface area to volume ratio. More rigid RBCs may be less able to squeeze through the capillaries, and they also increase platelet margination described above, both of which increase the susceptibility to thrombosis [18]. Increased rigidity can be caused by either a decrease in membrane deformability, determined primarily by the cytoskeleton and the cellular metabolic energy, or the cytoplasmic viscosity, determined mainly by the haemoglobin concentration [19].

A major, clinically significant feature of some inherited diseases is RBCs with reduced deformability. RBCs of patients with sickle cell disease have membranes that are stiffer than those of normal cells [20, 21]. In addition, the cells themselves become strikingly stiffer when the mutated haemoglobin polymerizes inside and sickles the cells [22]. Other diseases, including β-thalassaemia, haemolytic anaemias caused by RBC antibodies, and hereditary stomatocytosis, also commonly have RBCs with stiff membranes [23]. Some diseases, such as diabetes, hypertension, lower limb vein thrombosis and coronary heart disease, can secondarily alter the properties of RBCs, making them stiffer and prothrombotic [24]. Decreased RBC deformability reduces permeability of blood clots and thrombi, which may have implications for the penetration of fibrinolytic agents [25]. Stored RBCs exhibit altered biophysical characteristics, including higher cell rigidity that accounts in part for impaired blood flow haemodynamics and adverse effects of RBC transfusion [26].

Interaction of RBCs with platelets

RBCs can modulate platelet reactivity directly through either chemical signalling or adhesive RBC–platelet interactions. RBCs promote platelet aggregation and degranulation by releasing ATP and ADP under low pO_2, low pH and in response to mechanical deformation [27, 28]. Another mechanism for platelet activation by RBC lysate is extracellular haemoglobin, which enhances platelet activation by lowering NO bioavailability [29]. Cell-free

haemoglobin acts as a strong NO scavenger, preventing NO-mediated suppression of activated platelets [30]. Damaged RBCs also release arginase that cleaves L-arginine, a substrate for NO production [29]. In acute coronary syndrome, RBC transfusion increases platelet reactivity [31]. In the presence of RBCs, platelets are less responsive to aspirin, even when synthesis of thromboxane A_2 is inhibited [32]. When RBCs are damaged by high shear in continuous flow ventricular assist devices, free haemoglobin induces platelet aggregation, contributing to high risk of thrombotic complications [33].

RBCs can play a role in thrombus formation under flowing conditions at venous shear rates by direct adhesive interactions with platelets [34, 35]. The RBC–platelet adhesive interaction may be important in pathological conditions associated with a high incidence of thrombosis, such as thalassaemia [36] or sickle cell disease [37]. Interestingly, in the widely used ferric chloride *in vivo* model of thrombosis, platelets bind to the wall-associated RBC-derived material rather than the endothelium [38].

Interactions of RBCs with the endothelium

There is increasing evidence that RBCs can be incorporated into thrombi via specific interactions with activated endothelial cells and/or exposed subendothelial matrix. Normal mature RBCs do not interact with endothelium, but they become highly sticky under certain pathological conditions, and this adhesion of abnormal and/or stimulated RBCs to vascular endothelium can contribute substantially to microvascular occlusions associated with thrombosis. The most common pathological states in which RBCs interact with the endothelium include sickle cell disease [39], malaria [40] and diabetes [41]. Structurally and metabolically altered RBCs, which are present in higher numbers in RBCs that have been stored longer, have greater strength of adhesion to the endothelium [26].

Phosphatidylserine exposure in RBC membrane

Efficient blood coagulation requires sufficient prothrombotic surfaces for the proper assembly of the prothrombinase complex and generation of thrombin to initiate clotting. These surfaces are provided by cells that expose phosphatidylserine, a negatively charged phospholipid, which is normally on the cytoplasmic side of the membrane to separate this procoagulant surface from plasma coagulation factors [42]. Much of the focus on exposure of phosphatidylserine in coagulation has been on activated platelets, but recently, it has been shown that RBCs also are involved. Under conditions of apoptosis or RBC damage, such as high shear rates, inflammation or oxidative stress, RBCs can lose membrane asymmetry and expose phosphatidylserine [43]. Phosphatidylserine externalization and shedding are mediated by increased cellular Ca2+ flux and play an important role in natural RBC senescence [44]. Because of the large numbers of RBCs present in the blood, even a small fraction of RBCs with phosphatidylserine exposure can result in prothrombotic conditions. Even in healthy individuals, about 0·5–0·6% of the RBC population expresses phosphatidylserine and provides an active surface for prothrombin activation. This subpopulation of RBCs might account for up to 40% of the thrombin-generating potential of whole blood [45].

Some remarkable examples of phosphatidylserine exposure in RBC membranes are sickle cell disease and thalassaemia [46, 47]. The abnormal phosphatidylserine exposure in sickle cell disease is thought to result from the repeated cell sickling and unsickling that are linked to polymerization and depolymerization of mutated haemoglobin [48]. An increase in RBC phosphatidylserine exposure in β-thalassaemia patients has been shown to be connected with eryptosis, the suicidal death of RBCs [49].

RBC-derived microparticles

Activation, ageing and apoptosis of various cells, including RBCs, are accompanied by the formation of microscopic extracellular membranous structures named microvesicles or microparticles (MPs). The ability of cells to generate MPs *in vivo* is an important regulatory mechanism of physiologic reactions, a means for intercellular communications and a pathogenic component in many diseases that impact haemostasis and thrombosis [50, 51]. Formation of RBC-derived MPs is typical during the *ex vivo* storage of whole blood [52], and accumulation of MPs is thought to be responsible for an increased incidence of deep vein thrombosis after transfusion of 'old' red cells [53]. An increase in the number of circulating RBC-derived MPs has been found in RBC-related prothrombotic states, such as sickle cell disease and haemolytic anaemia [54]. Irrespective of their source, elevated plasma levels of MPs are associated with a reduced clotting time and a dose- and time-dependent increase in thrombin generation, suggesting that the MPs enhance hypercoagulability.

The ability of RBC-derived MPs to enhance thrombin generation has been associated with expression of phosphatidylserine [55] and possibly also tissue factor [56]. RBC-derived MPs are capable of activating coagulation by other clotting factors or supporting anticoagulant reactions. The circulating MPs can internalize free haem and transfer it to vascular endothelium, promoting vaso-occlusion, or amplify systemic inflammation via thrombin-mediated activation of the complement system [57].

Given the broad procoagulant activity of RBC-derived MPs, they are considered a potential agent for treatment of haemostatic disorders [58].

RBC storage

Stored RBCs undergo a complex structural and metabolic impairment that includes leakage of haemoglobin from the cells and haemolysis, reduced energy and NO production, formation of toxic products, such as lysophospholipids and free iron, phosphatidylserine exposure and shedding MPs [59]. All these and other changes that occur to RBCs during storage make infusions of RBCs a procedure with frequent side-effects and complications, including an increased incidence of deep vein thrombosis [53]. During the storage of RBCs, the MP concentration increases and the number of those that express phosphatidylserine also increases [60], which represents a mechanism by which stored RBCs could promote thrombotic complications after infusion. Another potential mechanism that underlies deleterious effects upon RBC infusion is NO scavenging by haemoglobin released from RBCs during storage [61].

RBC destruction (haemolysis)

More or less massive *in vivo* haemolysis with release of free, extracellular haemoglobin into the blood is the pathogenic basis of hereditary and acquired haemolytic anaemias of various aetiologies, of which immune haemolysis is the most common. These conditions are accompanied by (pro)thrombotic disorders that vary from laboratory signs of hypercoagulability to life-threatening complications, such as disseminated intravascular coagulation [62] and venous thromboembolism [63]. There are several pathogenic mechanisms by which haemolysis may lead to intravascular coagulation. First, damaged RBCs release free haemoglobin and haem that are toxic to many cells and tissues. Extracellular haemoglobin sequesters NO and thus promotes activation of endothelial cells and adhesion/aggregation of platelets [64]. Free haem upregulates haem oxygenase activity, generates reactive oxygen species and activates endothelial cells and macrophages directly [65]. Second, immune haemolysis is accompanied by production of TNF-α which induces tissue factor expression in endothelial cells and also decreases the endothelial expression of thrombomodulin, a potent modulator of thrombin activity [62]. Third, haemolysis results in a massive release of procoagulant RBC-derived MPs [66].

Interactions of RBCs and fibrinogen

The tendency of RBCs to form rouleaux under low shear conditions requires fibrinogen [67]. An increase in fibrinogen concentration can result in greater RBC aggregation, which is associated with a higher incidence of thrombosis. Such RBC aggregation has generally been considered to be caused by the non-specific binding of fibrinogen to RBC membrane. However, there is now some evidence for the existence of specific interactions between fibrinogen and an integrin receptor on the RBC membrane [68], either a β3 integrin [69] or CD47 (integrin-associated protein) [70] or both. Interestingly, RBCs from a Glanzmann's thrombasthenia patient (a rare hereditary bleeding disease caused by αIIbβ3 mutation) show impaired fibrinogen binding [69]. The probability of binding interactions of RBC and fibrinogen progressively decrease with *in vivo* cell ageing, likely associated with the loss of sialic acid on older RBCs [71]. The administration of fibrinogen concentrate, which is critical for the formation of a fibrin clot in the perioperative setting and in massive haemorrhage, may include interaction with RBCs followed by haemostatic effects of RBC aggregates.

Effects of RBCs on clot structure

The presence of RBCs affects the structure of fibrin clots. Intermediate RBC concentrations cause heterogeneity in the fibre network with pockets of densely packed fibres alongside regions with few fibres [72]. With high levels of RBCs, fibres are arranged more uniformly but loosely around the cells. There is also a significant increase in fibre diameter upon RBC incorporation, and the viscoelastic properties of the clot are influenced. Besides the effects of intact RBCs, free extracellular haemoglobin prolongs clotting time of fibrinogen due to impaired polymerization [73]. Therefore, intact or damaged RBCs trigger variability in fibrin network structure, individual fibre characteristics and overall clot viscoelasticity, which has important implications for *in vivo* clot formation, maturation, stability, embolization and the efficacy of prophylactic anticoagulation and therapeutic fibrinolysis [72, 74]. It has been shown that RBC retention within clots determines thrombus size dependent on factor XIIIa activity [75], a plasma transglutaminase that cross-links fibrin polymer covalently increasing its mechanical stability, via cross-linking of the fibrin α chains [76]. RBCs are incorporated into all types of clots and thrombi formed in whole blood, both *in vitro* and *in vivo*, either venous [77] or arterial [31].

RBCs and fibrinolysis

RBCs are an important component of the complex reactions in clot formation and thus determine the ultimate physical and biological properties of fibrin, which affect profoundly the course of its dissolution [78]. As a

generality, incorporation of RBCs increases the lytic resistance and decreases the permeability of fibrin in a dose-dependent manner [79, 80]. In addition, the RBC-induced retardation of fibrinolysis correlates with mechanical stabilization and strengthening of fibrin clots, which was shown for thrombi in experimental cerebral ischaemia [81].

RBCs in clot contraction

Clot contraction, or retraction, has been proposed to be involved in haemostasis to form a tighter seal to stem bleeding, to pull clots or thrombi closer to the vessel wall so that they are less obstructive, and in wound healing. Clot contraction requires platelets and fibrin or fibrinogen. Non-muscle myosin IIa inside the platelet interacts with actin filaments attached to the cell membrane integrin αIIbβ3 via talin and kindlin. Fibrin or fibrinogen binds to αIIbβ3 outside the platelet to link other platelets [82, 83].

Contracted blood clots develop a remarkable structure, with a meshwork of fibrin and platelet aggregates on the exterior of the clot and a close-packed, tessellated array of compressed polyhedral erythrocytes, named polyhedrocytes, within (Fig. 1). Platelets (with their cytoskeletal motility proteins) and fibrin(ogen) (as the substrate bridging platelets for contraction) are required to generate the forces necessary to segregate platelets/fibrin from RBCs and to compress these cells into a tightly packed array [84].

The structure and properties of contracted clots and the kinetics of contraction vary depending on the relative amounts of platelets, fibrinogen and RBCs and the conditions of clotting. Clot contraction occurs in three sequential phases, each characterized by a distinct rate constant. Thrombin, calcium ions, the integrin αIIbβ3, non-muscle myosin IIa, factor XIIIa cross-linking and platelet count all promote one or more phases of the clot contraction process. In contrast, RBCs impair contraction and reduce stiffness, while increasing the overall contractile stress generated by the platelet-fibrin meshwork [75, 76, 85].

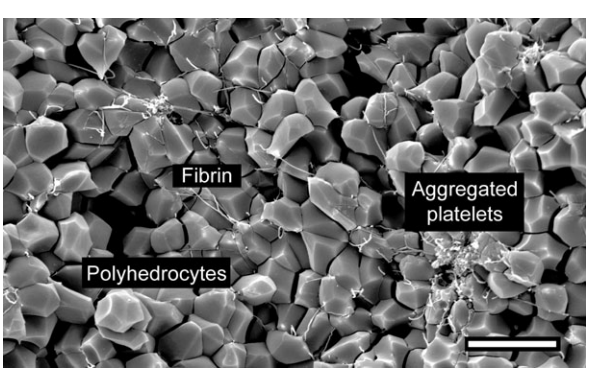

Fig. 1 The structure of contracted whole blood clot. Scanning electron micrograph of the interior of a contracted whole blood clot activated by thrombin following recalcification. The red blood cells are compressed by platelets pulling on fibrin to change shape from biconcave to polyhedral and are tightly packed; hence, they are named polyhedrocytes. A few fibrin strands and platelet aggregates are visible, but most of the platelets and fibrin are on the exterior of the contracted clot. Magnification bar = 10 μm.

Polyhedrocytes are the major component of venous clots, demonstrating that clot contraction occurs *in vivo* and suggesting that polyhedrocytes may play a role in haemostasis, at least on the venous side. Polyhedrocytes have also been observed in human arterial and especially venous thrombi, and pulmonary emboli, taken from patients [84, 86]. Moreover, the kinetics of contraction and extent of contraction can be different in patients with sickle cell disease, ischaemic stroke and deep vein thrombosis [87]. Such experiments suggest that the extent of clot contraction and the prevalence of polyhedrocytes may be associated with thrombosis and could be a marker of prothrombotic conditions.

Conclusions

The best-known effects of RBCs in clotting *in vivo* are rheological, involving laminar shearing with platelet margination plus aggregation and deformability of RBCs. In addition, RBCs interact directly and indirectly with endothelial cells and platelets, which may be involved in thrombosis. Both the stiffness of RBCs and the extent to which they form a procoagulant surface to generate thrombin through exposure of phosphatidylserine appear to play an important role. RBC-derived MPs transfused with stored RBCs or formed in various pathological conditions associated with haemolysis have strong procoagulant potential along with prothrombotic effects of the extracellular haemoglobin and haem. RBCs directly interact with fibrin(ogen) and affect the structure, mechanical properties and lytic resistance of clots and thrombi. Finally, the results on clot contraction demonstrate how contracted clots form an impermeable barrier made of tessellated polyhedral RBCs (polyhedrocytes) important for haemostasis and wound healing and to restore flow past obstructive thrombi. In summary, RBCs may perform a dual role, both helping to stem bleeding but at the same time contributing to thrombosis in several ways.

Funding

Some of the authors' work mentioned here was supported by NIH grants NHLBI HL090774 and UO1HL116330 and NSF grant DMR1505662.

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