ENHANCEMENT OF PLATELET ACTIVATION AND AGGREGATION BY ERYTROCYTES: ROLE OF RED CELLS IN THROMBOSIS

By

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Enhancement of platelet activation and aggregation by erythrocytes: 
role of red cells in thrombosis

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Abstract

The exact role of erythrocytes in thrombosis is unknown. Erythrocytes have long been considered simple cells, full of hemoglobin and devoid of a nucleus or mitochondria, their only purpose being to deliver oxygen to bodily tissues. Although this is true, and functionally important, it is becoming increasingly clear that erythrocytes may contribute more to hemostasis than previously thought. Erythrocytes have recently been implicated in processes related to thrombosis and blood clotting. Recent research focusing on this “simple cell” has led us to believe that erythrocytes undergo a process of programmed cell death termed “eryptosis” during senescence. This process is similar to apoptosis of nucleated cells.

Under normal circumstances, the plasma membrane of blood cells is maintained in an asymmetric state. However, during various physiologic and pathophysiologic conditions there is a loss of membrane phospholipid asymmetry such as: i) platelet
activation, ii) apoptosis, and iii) eryptosis (programmed cell death of erythrocytes). Common to each of these is the appearance of phosphatidylserine (PS) on the outer leaflet of the plasma membrane. Exposure of PS provides an attachment site for the prothrombinase complex as well as signals a cell for recognition and uptake by macrophages. PS displayed on the surface of erythrocytes signals the cell for phagocytosis, however, we postulate that the period of time between PS exposure and clearance by macrophages could enhance thrombin formation. An increased incidence of thrombosis due to the interaction between platelets and erythrocytes could lead to clinically significant implications in aspirin resistance, blood banking, and disease states associated with increased risk of thrombotic episodes.

**Introduction**

Diseases involving blood clotting are among the leading causes of death in United States.¹ These diseases involve thrombosis and formation of a clot which leads to obstruction of a blood vessel and ischemia. Included among these thrombotic diseases are: stroke which affects the brain tissue, myocardial infarction (heart attack), and deep vein thrombosis. According to the American Heart Association, approximately 1.2 million people have a heart attack each year, of which 452,000 are fatal. In addition, stroke is the third largest cause of death, affecting approximately 700,000 people each year.² Heart disease, also known as coronary artery disease, is the leading cause of death
in America and is caused by inflammation and atherosclerosis (the buildup of fatty plaques within a blood vessel). Therefore, heart disease puts individuals at higher risk for the occurrence of atherothrombotic events such as heart attack, stroke, and deep vein occlusion. These events frequently lead to death or permanent physical disability.

Thrombosis occurs due to the formation of a clot (platelets and fibrin) within a blood vessel that subsequently leads to occlusion. Antithrombotic therapy, such as the administration of salicylic acid (aspirin), can be valuable in the prevention of these events. A daily aspirin dose between 50-100 mg/d can inhibit platelet activation and subsequent thrombin formation, leading to a reduced risk of thromboembolic events in these individuals. However, it is becoming increasingly clear that platelets are not the only constituents responsible for thrombosis. It has been suggested that erythrocytes actively participate in thrombosis. In addition, the externalization of phosphatidylserine on the outer erythrocyte membrane has been shown to cause increased blood coagulation, endothelial adherence, and cell-cell recognition. Investigation into the role of erythrocytes and thrombosis, their interaction with platelets, and possible thrombin production would be of great clinical significance.

**Erythrocytes**

Erythrocytes (red blood cells) are small, hemoglobin filled cells which occupy approximately 10% of the total blood volume. Although these cells are anucleate and lack
mitochondria, they perform the important task of delivering oxygen to body tissues and transporting carbon dioxide to the lungs. For ATP production, the erythrocyte relies exclusively on the degradation of glucose, it does not use the oxygen it carries for energy. Under normal circumstances erythrocytes have a lifespan of approximately 120 days in which they perform their various functions until they undergo senescence and cell death. Senescent erythrocytes are detected and removed from the blood by the spleen and reticuloendothelial system. The biconcave shape of the healthy erythrocyte permits flexibility and a large surface area, thus allowing for efficient travel through blood vessels and perfusion through capillaries.

Erythrocytes are composed of the protein hemoglobin and an elaborate plasma membrane consisting of an array of lipids and proteins. The dynamic structure of the red cell membrane has been attributed to disorders such as hereditary stomatocytosis, hereditary spherocytosis, hereditary elliptocytosis, and hereditary pyropoikilocytosis, which result in hemolytic anemia. Hemolytic anemia is a consequence of the premature breakdown of erythrocytes due to hemolysis (cell bursting). While the main function of the erythrocyte is oxygen transport, new evidence has led us to believe that erythrocytes perform other functions as well. Recent research has shown that erythrocytes may also play a role in vasodilation and antimicrobial defense during an immune response.
Platelets

Platelets are small (2-3 µm in diameter), irregularly shaped fragments of much larger megakaryocytes and have a lifespan of 9-12 days. Similar to erythrocytes, platelets lack a nucleus. Structurally composed of a dynamic cell membrane and cytoskeleton, platelets also contain secretory granules consisting of various cytokines (platelet activation factor), growth factors, coagulation proteins, and adhesion molecules. Platelets play a crucial role in the maintenance of hemostasis and participate in the stimulation of the coagulation cascade. Under normal circumstances, platelets travel along the surface of blood vessel walls in an inactivated state. Upon contact with a damaged blood vessel, platelets demonstrate a high tendency for adherence. Adherence of platelets within a damaged blood vessel can lead to narrowing of the vessel and possible thrombosis.

Platelet Activation

Platelets are activated upon contact with tissue factor or a collagen-exposing damaged blood vessel. Upon activation, platelets undergo various physical and chemical changes including the formation of pseudopods, the release of platelet microparticles (PMPs), and exposure of phosphatidylserine on the outer leaflet of the plasma membrane. Negatively charged phosphatidylserine provides an assembly site for the
prothrombinase complex (factors Xa, Va, and Ca^{2+}) which allows for the conversion of prothrombin to thrombin.\textsuperscript{15,17} Thrombin is an important protein that is released freely into the bloodstream to convert fibrinogen to fibrin. Fibrin is a fibrous protein that contributes to the formation of a “mesh” surrounding the site of injury and subsequent development of a clot.\textsuperscript{4} Excessive thrombin and clot formation can lead to thromboembolism and the occurrence of myocardial infarction, stroke, or deep vein thrombosis.

**Apoptosis**

Nucleated cells are regulated and die by a process of programmed suicidal cell death termed apoptosis.\textsuperscript{18} Apoptosis of cells occurs under normal circumstances in tissues such as the skin, gut, and immune system where the balance of cell death and replacement are of great importance. For example, the differentiation of fingers and toes during embryonic development relies on the process of apoptosis. In contrast, another form of cell death which does not occur under normal circumstances is called necrosis. Necrosis results due to cellular injury and trauma to tissues, such as ischemia or infection, and can be detrimental to the host due to the release of cellular constituents into surrounding tissue.\textsuperscript{19}

Apoptosis is regulated by a group of cysteine proteases called caspases and mediated by two major signaling pathways: (1) an ‘extrinsic pathway’ via Fas or TNF receptors and (2) an ‘intrinsic pathway’ via mitochondrial release of cytochrome c.\textsuperscript{4}
Characteristics of apoptosis include cell shrinkage, pyknosis (chromatin condensation/nuclear degeneration), zeiosis (membrane blebbing), phosphatidylserine exposure, and the formation of apoptotic bodies.\textsuperscript{18,19} Under normal circumstances, the phospholipid phosphatidylserine is restricted to the inner leaflet of the plasma membrane. However, in the early stages of apoptosis phosphatidylserine “flips” and becomes exposed on the outer leaflet of the lipid bilayer. Exposure of phosphatidylserine on the exterior surface of the cell acts as a signal for uptake by macrophages.\textsuperscript{9}

**Eryptosis**

Recently, it has been demonstrated that erythrocytes undergo a process similar to apoptosis during senescence termed “eryptosis”. Eryptosis, the programmed death of erythrocytes, is characterized by cell shrinkage, membrane blebbing, activation of proteases, and phosphatidylserine exposure.\textsuperscript{20} This “activation of the red cell” provides a mechanism for the red cell to escape hemolysis and is the consequence of pathways activated by various stressors (e.g. oxidative stress) and mediators (PAF, PGE\textsubscript{2}).\textsuperscript{21} Exposure of phosphatidylserine on the outer membrane of the red cell signals for recognition, uptake, and degradation by macrophages.\textsuperscript{9,22} Research has shown many initiators of programmed erythrocyte death including curcumin, retinoic acid, zinc, cadmium, and others.\textsuperscript{23-26}
Two signaling pathways elicit the activation of eryptosis: (1) opening of \( \text{Ca}^{2+} \) channels following subsequent formation of prostaglandin \( \text{E}_2 \) and (2) release of platelet activating factor (PAF) by phospholipase \( \text{A}_2 \), which leads to the activation of a sphingomyelinase and formation of ceramide.\textsuperscript{22,27} These pathways ultimately lead to phosphatidylserine exposure and clearance by macrophages, however, the period of time between these events should be considered as the characteristics of eryptosis are similar to processes of both platelet activation and apoptosis (Table 1).

**Table 1: Comparison of platelet activation, eryptosis, and apoptosis**

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<th>Platelet Activation</th>
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<tr>
<td><strong>Activation mechanisms</strong></td>
<td>Exposure to tissue factor and collagen;</td>
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<td><strong>Characteristics</strong></td>
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<td>Phosphatidylserine exposure, cell shrinkage, membrane blebbing, activation of proteases</td>
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<tr>
<td><strong>Consequence of PS exposure</strong></td>
<td>Assembly of the prothrombinase complex</td>
<td>Recognition and removal by macrophages</td>
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<td>(Signaling Pathways)</td>
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<td>Phosphatidylserine exposure, cell shrinkage, pyknosis, membrane blebbing, formation of apoptotic bodies</td>
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Biochemical signaling pathways of both platelet activation and cryptosis involve release or formation of platelet activating factor (PAF). PAF activates both platelets and erythrocytes, implicating that these two events could be interrelated under certain conditions. In platelets, exposed phosphatidylserine provides an attachment site for the prothrombinase complex. The exposure of phosphatidylserine on the outer leaflet of the red cell membrane may, like platelet activation, interact with the prothrombinase complex and lead to the formation of thrombin.

**Discussion**

Until recently, erythrocytes have been characterized as little more than carriers of oxygen and carbon dioxide. Although this function is vital to the existence of aerobic organisms, recent research has shown that the erythrocyte also exhibits other functions throughout its lifetime. Of clinical importance is the active role that erythrocytes play in hemostasis and thrombosis.⁶ Phosphatidylserine exposure on the outer leaflet of the plasma membrane has been proposed as a mechanism leading to increased adherence of the erythrocyte and impedance of the microcirculation.⁸ In addition, a number of diseases such as sickle cell disease, β-thalassemia, and hereditary hemolytic anemia have implicated PS-exposing erythrocytes as a mechanism for the increased risk of thrombosis seen in these disorders.⁶,²⁸-³⁰
Alteration of erythrocytes is also seen during blood cell storage (blood banking). Blood is routinely stored for up to 42 days under cold storage conditions. Blood banking has been shown to effect erythrocytes by increasing phosphatidylserine exposure in addition to increasing its rigidity and adherence to endothelial cells.\textsuperscript{31} These alterations in the erythrocyte membrane may pose increased hemodynamic risk to patients receiving blood transfusions.

Furthermore, erythrocyte activation may play a role in aspirin resistance. Aspirin is an anti-platelet drug. It acts as a cyclooxygenase inhibitor, thus preventing the formation of thromboxane \textit{A}_2 and subsequent platelet activation. However, aspirin is not always affective at preventing a thrombotic event. Aspirin resistance is described as the inability of aspirin to: (1) protect patients from ischemic vascular events; (2) produce an anticipated effect on one or more tests of platelet function; (3) inhibit biosynthesis of thromboxane (TX); or (4) cause a prolongation of the bleeding time.\textsuperscript{32} Laboratory aspirin resistance has been reported in 60-80\% of patients after acute coronary syndromes.\textsuperscript{33} In their editorial on aspirin resistance, Hankey and Eikelboom suggest erythrocyte activation of platelets as an alternative pathway of platelet activation.\textsuperscript{34} This pathway would not be blocked by aspirin, and could potentially stimulate receptors on platelets and contribute to the coagulation cascade.
**Conclusions**

The interaction between platelets and erythrocytes along with their combined role in thrombosis has not been thoroughly investigated. Our hypothesis is that erythrocyte activation will contribute to greater thrombin formation and activity rather than that of platelets alone. Future research should be conducted in order to explore the role of red cells in thrombosis and platelet activation. If results revealed increased thrombin production, insight could be obtained as to whether or not erythrocytes are active contributors to cardiovascular events and aspirin resistance.
References


Erythrocytes (red blood cells) are small, hemoglobin-filled cells which occupy approximately 10% of total cell volume. These cells, although lacking mitochondria and a nucleus, perform the important function of delivering oxygen to body tissues and transporting carbon dioxide to the lungs for excretion. Their biconcave shape and flexibility allow for quick travel within blood vessels while maintaining the integrity of both the cell and vessel wall. Although the main purpose of the erythrocyte is to carry oxygen, new data leads us to believe that they are involved in other activities as well. Recent research suggests erythrocytes play roles in attacking bacteria [1] and vasodilation [2-3]. Under normal circumstances these various functions are performed throughout the ~120 day lifespan of the cell until senescence and cell death.

Nucleated cells are regulated by a process of programmed cell death called apoptosis. Characteristics of apoptosis include cell shrinkage, pyknosis (chromatin condensation/nuclear degeneration), membrane blebbing, the formation of apoptotic bodies, and phosphatidylserine exposure [4,5]. Phosphatidylserine expression on the outer leaflet of the cell membrane acts as a signal for uptake by macrophages [4,5]. In many cases, apoptosis depends on activated proteolytic caspases (cysteine proteases) for regulation.

Recent research has shown that anucleate platelets undergo an apoptosis-like event during activation [6-8]. Activated platelets express phosphatidylserine on the outer leaflet of their plasma membrane which provides an assembly site for the prothrombinase complex. Attachment of the prothrombinase complex ultimately leads to the formation of thrombin.

Recently, it has been demonstrated that erythrocytes also undergo an apoptosis-like process termed “erythroptosis” during senescence. Erythroptosis, or more commonly “eryptosis”, is a process characterized by cell shrinkage, membrane blebbing, and phosphatidylserine exposure. Similarly to nucleated cells, the exposure of phosphatidylserine allows for subsequent uptake and disposal by macrophages [9].
Erythroptosis is initiated by various stressors and two signaling pathways. The consequent steps of these pathways leads to increased cytosolic calcium and ceramide formation, ultimately causing phosphatidylserine exposure [10-11]. Common to both erythroptosis and platelet activation is phosphatidylserine exposure. Although the primary response to phosphatidylserine exposure on red cells seems to be clearance by macrophages, we postulate that the period of time between PS exposure and clearance could enhance the formation of thrombin.

Thrombosis due to red blood cells may be caused by various mechanisms [12]. First, PS exposure may cause shape changes and consequently lead to increased cell-cell interactions and adherence to endothelial cells [13]. Of clinical importance is the alteration of erythrocytes during blood cell storage (blood banking). The effect of blood banking has been shown to increase the rigidity and endothelial cell adherence of the erythrocyte in addition to increasing phosphatidylserine exposure [14,15]. The second mechanism is the role that PS plays in the formation of thrombin. These combined effects may ultimately lead to the impairment of blood flow. Furthermore, erythrocyte activation may play a role in aspirin resistance. Erythrocyte activation, and possible thrombin formation, may lead us to believe that erythrocytes are more to blame for aspirin resistance rather than platelets alone. All of these factors discussed are of great clinical significance and support further investigation into the role of eryptosis in thrombosis.

References


