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Role of Cellular Elements in Thrombus Formation and Dissolution

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Abstract

Although fibrin forms the core matrix of thrombi, their structure depends also on the cellular elements embedded in its meshwork. Platelets are essential in the initial stages of thrombus formation, because they adhere and aggregate at sites of blood vessel wall injury and then serve as a surface for coagulation reactions, the overall rate of which determines the final structure of fibrin. In addition, platelets affect fibrinolysis through their proteins and phospholipids, which modulate plasmin activity. Leukocytes form mixed aggregates with platelets and thus influence the structure of thrombi. After activation they secrete different proteases (elastase, cathepsin G, matrix metalloproteinases) that enhance the von Willebrand factor-dependent platelet adhesion. Leukocyte-derived enzymes, first of all elastase, effect fibrinolysis by direct digestion of fibrin or indirectly modulate it by partial degradation of zymogens and inhibitors of coagulation and fibrinolytic proteases.

Keywords

platelets; leukocytes; von Willebrand factor; neutrophil elastase; matrix metalloproteases

INTRODUCTION

Thrombi are complex structures that are composed not only of fibrin meshwork, but also contain blood-borne cellular elements like platelets, leukocytes and red blood cells. Platelets play an essential role in the initial response to vascular injury as they adhere to vessel wall components, become activated, aggregate and secrete mediators that promote platelet activation and also attract leukocytes. In addition to the plug formation, which transiently stops bleeding, platelets provide surface for the subsequent steps of the coagulation cascade leading to fibrin formation. Blood coagulation complexes function only in compartments and platelets serve the phospholipid surface for these reactions (recently reviewed in [1]). Platelet adhesion depends on the shear rate and is influenced by proteases that are generated during the activation of the coagulation cascade or originate from cellular elements. Ultimately the thrombin activity and the platelet-dependent transglutaminase affect the structure of the fibrin network and thus the architecture of the thrombus that is formed later on. Platelets influence not only thrombus formation, but also fibrinolysis. PAI-1 (plasminogen activator inhibitor-1), phospholipids and myosin from platelets modulate fibrinolysis (for recent review see [2]). Leukocytes migrate into the thrombus and following activation, they secrete different enzymes: serine proteases, matrix metalloproteases. These enzymes modulate platelet adhesion and also fibrinolysis. Neutrophil elastase promotes an

alternative pathway of fibrinolysis [3] both *via* direct degradation of fibrin and effects on the plasminogen-plasmin system (reviewed in [4]). Red blood cells may seem passive participants being just entrapped in thrombi, but actually they also influence hemostasis by secreting pro-coagulant substances and contributing to the phospholipid surface for assembly of pro-coagulant complexes.

The relative abundance of each cell type within the fibrin structure is influenced primarily by hemodynamic factors, thus explaining why the composition of arterial thrombi differs from that of venous thrombi. An arterial thrombus develops under high shear and is primarily composed of platelet aggregates in a reticulum of fibrin. A venous thrombus develops under conditions of slow blood flow or stasis and is primarily composed of red blood cells in a fibrin network with relatively few platelets. Some leukocytes are found early during thrombus formation, others are recruited by chemotactic agents released by aggregating platelets and are entrapped in the thrombus (reviewed in [5]).

PLATELETS

The inner lining of normal blood vessels is composed of endothelial cells that form a surface resistant to the adhesion of circulating platelets. In areas where the endothelium is altered or at sites of vascular damage firm platelet attachment rapidly occurs. In response to hemorrhage, circulating platelets adhere to exposed subendothelial tissues and then recruit additional platelets into aggregates that function as pro-coagulant surfaces. The hemostatic response to vascular injury is contingent on the extent of damage, the specific matrix proteins exposed and shear stress.

Under high shear stress platelets move rapidly on von Willebrand factor (vWf) that is bound to collagen on subendothelial surfaces. The highest wall shear rate in the normal circulation occurs in small arterioles of 10 to 50 μm in diameter, in which shear rates have been estimated to vary between 500 and 5000 s^{-1} [6]. Values up to 10 times higher have been calculated to occur at the tip of severe stenosis in atherosclerotic coronary arteries [7, 8]. vWf is constitutively bound to the subendothelial matrix and supports platelet adhesion when exposed to flowing blood [9]. The binding of soluble vWf to non-activated platelets is tightly regulated to prevent aggregation in the circulation, but vWf immobilized onto a surface is highly reactive toward flowing platelets. Circulating vWf multimers are in coiled conformation that shields the A1 domain from interacting with platelets, whereas binding to a substrate under shear stress extends the shape of the molecule [10]. vWf binds collagen type I and III through its A3 domain [11], whereas the A1 domain is a putative collagen type VI binding site [12]. vWf molecules form high-strength bonds with GPIIb α receptor on platelets that is the major platelet receptor for vWf [13]. At high or pathological shear the GPIIb α -vWf interaction is necessary to slow down platelet velocity sufficiently to enable GPVI-collagen-mediated platelet signaling, which leads to the activation of GPIIb/IIIa. This interaction has high resistance to tensile stress, but a limited half-life. While kept in close proximity to the surface and in slow motion, platelets can form stabilizing bonds that would not occur directly in rapidly flowing blood. Under low shear stress ($<500 \text{ s}^{-1}$) vWf is not required for the initial platelet-surface interaction. GPIIa and GPVI platelet collagen receptors have a defined role in thrombus formation. GPVI belongs to the immunoglobulin superfamily and is non-covalently associated with the FcR γ chain, which serves as the signal-transducing part of the receptor [14]. GPIIa is an integrin collagen receptor and it has been proposed that its binding to collagen facilitates the engagement of GPVI, thus GPIIa plays a supportive rather than an essential role in platelet interactions with native, fibrillar collagen. GPVI is a low-affinity and signal-transducing receptor and its function is not impaired in the absence of GPIIa. It is considered to play a crucial role in platelet activation [15]. GPVI-collagen interaction is a prerequisite for integrin-mediated adhesion

[16]. Resting platelets express integrins in a low-affinity binding state to avoid interaction with fibrinogen or plasma fibronectin [17]. When platelets become activated their integrins shift to a high-affinity state and bind ligands [16].

Thrombogenic surfaces interact only with the initial layer of adherent platelets, but propagation of the resulting activating signals promotes the binding of soluble adhesive proteins and thrombus growth. These signals originate from the costimulation of platelet receptors by soluble agonists that become available from platelets themselves and by the activation of the coagulation factor cascade. ADP, thrombin and thromboxane A₂ are of particular prominence in the process that turns GPIIb/IIIa into a high-affinity receptor capable of binding soluble adhesive proteins as a prerequisite for platelet aggregation. Both fibrinogen and vWf act as adhesive proteins and mediate platelet aggregation depending on the flow conditions. The subsequent interaction of multimeric vWf with both GPIIb/IIIa and activated GPIIb/IIIa may temporarily stabilize interplatelet contacts and allow the ensuing permanent bridging mediated by fibrinogen binding across activated platelet membranes. In the absence of fibrinogen thrombi are unstable, because fibrinogen along with vWf serves as the major ligand interconnecting platelets within the platelet plug [18, 19].

The adhesion of platelets at high shear rate is localized to the adventitia layer of the vessel wall, the media layer is resistant to vWf-dependent platelet adhesion despite the presence of fibrillar collagen. The vWf-binding sites of collagen are probably shielded by other vessel wall components. Serine-proteases causing enzymatic cleavage of vessel wall proteins and glycosaminoglycans increase the platelet coverage of the media layer [20]. Such serine proteases are plasmin and thrombin, which are generated *in vivo* at the site of vessel injury, or elastase and cathepsin G derived from recruited neutrophil leukocytes. The number of adherent platelets influences the growth of thrombi. They not only plug the damaged vessel temporarily, but also localize subsequent procoagulant events to the injury site by exposing phospholipids on the cell surface (first of all phosphatidylserine and phosphatidylethanolamine) and sustain the pro-coagulant response by protecting the bound coagulant enzymes from inhibition.

An essential pro-coagulant function of platelets is related to the tissue factor (TF) stored in their α -granules. In response to activation platelet TF is translocated to the cell surface by fusion of the α -granules with the plasma membrane, where it forms complex with factor VIIa and initiates coagulation. At the same time activated platelets are known to secrete tissue factor pathway inhibitor (TFPI), the major physiologic antagonist of the initiation of coagulation. Thus, efficient function of platelet TF requires elimination of TFPI, which may be evoked by interplay with co-localized leukocytes. Neutrophil surface proteases are known to degrade TFPI, thereby unleashing the start of coagulation [21]. Platelets are capable of translating gene transcripts into mature proteins and could thus in principle generate TF *de novo*. Alternatively, they can acquire TF *via* microparticle transfer, a process by which cell membrane proteins are exchanged between different cell types. TF expressing microparticles fuse with activated platelets, consequently inserting the protein into the platelet plasma membrane [22]. Microparticles are exocytotic products of the plasma membrane generated during cell activation, apoptosis, cell maturation, and mechanical stress. Microparticles circulate in blood under physiologic conditions and are supposed to originate preferentially from platelets. Circulating microparticles are heterogeneous in size, ranging between 0.1 μm and 1.0 μm . Their upper size limit may therefore be almost that of a platelet. In response to vascular injury *in vivo*, microparticles are rapidly recruited to the lesion site, triggering coagulation in a TF-dependent way [23, 24].

The number of platelets has striking effects on clot structure and hence on fibrinolysis. Platelet-rich plasma clots have regions with dense platelet aggregates, from which fibrin

fibers originate and they are resistant to fibrinolysis [25]. The fibers adjacent to the platelet aggregates are both thinner and denser than those devoid of platelets [25]. Platelet-rich clot lysis is suppressed because of stronger clot-retraction and thus reduced t-PA binding capacity owing to the high-density of retracted fibrin-fibres. As platelets contract, fibrin strands are pulled, and force is transmitted to the clot surface, resulting in clot retraction, so the clot will be a more resistant structure against mechanical forces [26]. Besides, fibrinolysis appears heterogeneous, proceeding through meandering channels and leaving platelet-rich areas unlyzed [25].

Platelet-derived phospholipids form a diffusion barrier to the thrombolytic agents and also bind some of them. Purified platelet phospholipids and synthetic phospholipids retard *in vitro* tPA-induced fibrinolysis through effects on plasminogen activation and plasmin function. The inhibition of plasminogen activation on the surface of fibrin correlates with the fraction of anionic phospholipid. After activation platelets release their cytosolic content and lose the majority of their phospholipid [27, 28]. Myosin, a principal structural cellular protein is also released and it interacts with the fibrin fibers masking their cofactor and plasmin-cleavage sites [29]. Myosin is abundant in thrombi, it is expressed in platelets, skeletal-, smooth- and cardiac muscle cells which are all important participants at sites of trauma, infarction or inflammation. Myosin binds both tPA and plasminogen and accelerates plasminogen activation, but only in free form (and not in complex with fibrin). It is not only a cofactor for plasminogen activation but also a substrate for plasmin and thus through its competition for the protease myosin can protect fibrin against lysis. Plasmin bound to myosin is thought to be protected against the inhibitor system of fibrinolysis similarly to fibrin-bound plasmin [30]. Platelets also influence fibrinolysis by secreting inhibitors: in addition to inhibiting plasmin, α_2 -plasmin inhibitor competes with fibrin for lysine binding sites of plasminogen, thus preventing the positive feed-back role of fibrin in plasminogen activation (reviewed in [31]). The secretion of plasminogen activator inhibitor-1 (PAI-1) is triggered by thrombin, thus inhibiting the conversion of plasminogen to plasmin by plasminogen activators (t-PA or urokinase). The inhibitory effect of PAI-1 is reduced when fibrin, plasmin and t-PA exist as a ternary complex [32]. Platelets contain cellular factor XIII and they also promote fibrin crosslinking by plasma factor XIII, thus contributing to generation of fibrin structure, which is more resistant to lysis (see review of Muszbek *et al.* in this issue).

In summary platelets set the stage for thrombus formation and growth and they prevent premature fibrinolysis.

LEUKOCYTES

Platelets interact with neutrophils through P-selectins and β_2 and β_3 integrins (for recent review see [33]). In resting platelets selectins are stored in the membranes of α -granules and upon activation they are redistributed to the platelet surface and initiate adhesion to leukocytes. Besides capturing neutrophils, platelets also secrete neutrophil and endothelial activators inducing production of inflammatory cytokines and release of neutrophil granules including proteases (serine proteases and matrix metalloproteases) [34].

Leukocytes are in a resting, low-adhesive state while circulating in blood. Stimulatory signals promote the migration of these cells through the endothelial layer (reviewed in [35]). This migration proceeds through a number of distinct steps, including initial rolling to slow down the leukocytes followed by firm adhesion to allow transendothelial migration. Several endothelial and leukocyte receptors have been identified that contribute to these processes. Leukocyte rolling over the endothelial layer is mediated by selectins which are exposed on the activated endothelial surface. They interact with the PSGL-1 (P-selectin glycoprotein

ligand-1) in the leukocyte membrane. The activation of signaling pathways bring leukocytes into a high-adhesive state required for stable adhesion. β_2 -Integrins are the most important players in this process. The major counter-receptors for the β_2 -integrin isotypes are the intercellular adhesion molecules (ICAMs), although many other proteins have the capability of interacting with this integrin-family [36].

Independently of their interaction with platelets neutrophils can directly bind to vWf through PSGL-1. This means that vWf can function as an adhesive molecule for leukocytes in a manner analogous to the vWf-platelet interaction. vWf recruits leukocytes into thrombi by mediating both rolling and stable adhesion of the cells [36]. Circulating neutrophils express only a few receptors on their surface and the secretory granules are sources of further receptors, which appear upon stimulation of the cell. L-selectin and the binding partner of the endothelial P-selectin are both expressed on the tips of microvilli of circulating neutrophils [37]. When circulating neutrophils are captured by the selectins presented by activated endothelium, fusion of secretory vesicle membranes with the plasma membrane is triggered [38], which causes immediate upregulation of neutrophil β_2 -integrins and chemotactic receptors.

Traditionally neutrophil granules are subdivided into peroxidase-negative (called also specific or secondary) and peroxidase-positive (primary or azurophil) granules based on the presence of myeloperoxidase. Azurophil granules contain serine proteases (elastase, cathepsin G and proteinase-3). The specific granules store matrix metalloproteinase-8 (MMP-8, known also as neutrophil collagenase). Matrix metalloproteinases are zinc-dependent endopeptidases, which are involved in the physiological and pathological remodeling of the extracellular matrix. They are capable of degrading various extracellular matrix proteins, but also can process a number of bioactive molecules. The MMPs share a common modular structure. The three common domains are the propeptide, the catalytic domain and the haemopexin-like C-terminal domain, which is linked to the catalytic domain by a flexible hinge region. The MMPs are synthesized as inactive zymogens with a propeptide domain that must be removed for activation. Neutrophils contain additional small storage granules named gelatinase granules based on their MMP-2 and MMP-9 content [39-41]. Upon stimulation the protease containing granules are released and their enzymes are able to influence blood coagulation and fibrinolytic components [3]. MMP-2 is able to cleave thrombin yielding an enzyme that lacks clotting and platelet-stimulating activity [42]. Elastase degrades factor XIII and inactivates factors VII, VIII, IX, and XII [43, 44]. In addition, elastase, cathepsin G and proteinase-3 are likely to modify the vessel wall structure, thus promoting platelet adhesion to the media layer [20]. Elastase degrades collagens, proteoglycans and particularly elastin. Cathepsin G is likely to digest proteoglycans. Both elastase and cathepsin G are able to activate proMMP-2 and MMP-2 then degrades extracellular matrix components, among others collagen type I, IV, IX, proteoglycans, laminin, fibronectin, gelatin [45].

Several MMPs are expressed in platelets including MMP-1, MMP-2, MMP-9 [46-48]. These platelet MMPs affect specific cellular receptors. For example, while platelet activation causes the expression of CD40L [49] and P-selectin [50] on the platelet surface, platelet MMPs cleave these proteins generating soluble forms. MMP-induced cleavage of GPIIb/IIIa has been reported [51]. After platelet activation MMPs cleave GPVI. Platelets with decreased levels of GPVI are less responsive to collagen. The data evidence that MMP-mediated GPVI cleavage provides a physiological mechanism to reduce platelet responsiveness to collagen [52]. Reduction in GPVI levels affects thrombus formation not only on collagen, but also on vWf, as GPVI-deficient platelets adhere less to vWf under flow conditions [53]. On the other hand P-selectin is shed from the surface of activated platelets by MMPs, generating a soluble product with procoagulant activity [54, 55].

Neutrophil elastase plays a dual role in fibrinolysis; it is able to degrade directly fibrin and it stimulates an alternative pathway of plasminogen activation. Elastase generates larger fibrin degradation products than plasmin, which differ in their effects on the clot stability from the effects of the plasminic products, and thus elastase digestion confers a specific pattern of dissolution under flow conditions [56]. When exposed to shear forces, fibrin digested by clot-embedded plasmin abruptly disassembles with concomitant release of large particles in the circulating phase. Such a disassembly is not observed with neutrophil elastase, which continuously releases soluble (and not particulate) products in the fluid phase. Because this difference in the dissolution pattern of fibrin with clot-embedded plasmin and elastase is seen even under the low shear rates typical for the venous circulation, it suggests a link between plasmin digestion and *in vivo* thromboemboli and lack of such interrelation in the case of elastase digestion [56].

In the alternative pathway of plasminogen activation neutrophil elastase cleaves plasminogen, which is then converted to des-kringle₁₋₄ plasminogen (mini-plasminogen) by removing the first 4 kringle domains from the native molecule. Mini-plasminogen is more readily activated to miniplasmin by plasminogen activators and no cofactors are needed for the activation [57-59]. Miniplasmin is more efficient on cross-linked fibrin than plasmin and it is ten-times less sensitive to inhibition by α_2 -antiplasmin than plasmin [58]. These effects of elastase on the function of the plasminogen/plasmin system can explain how *in vivo* elastase increases the global plasma fibrinolytic potential as reported for patients with pulmonary thromboembolism [60].

In summary, through their interactions with platelets and blood vessel wall neutrophil leukocytes can promote thrombosis, whereas through their effects on the classic fibrinolytic system they support fibrin dissolution.

RED BLOOD CELLS

Red blood cells (RBC) are not only innocent bystanders in hemostasis, a couple of roles have been described concerning their contribution to thrombus formation. Clinical observations suggest that a variety of bleeding disorders can be treated by elevation of RBC counts, even when platelet levels decrease or remain unchanged [61-63]. Increased RBC counts predispose to thrombosis. The simplest explanation is the elevation of blood viscosity, which leads to obstruction. High hematocrit increases the residence time of circulating platelets and coagulation factors near the activated endothelium, because it promotes the transport of platelets toward the vessel wall, thereby increasing their collisions with the vasculature [64, 65]. Besides effecting elevated viscosity, RBCs perform a chemical signaling role in hemostasis. They promote platelet aggregation and degranulation by releasing ATP and ADP under low pO₂, low pH and in response to mechanical deformation [66, 67]. Erythrocytes contribute to the activation of the coagulation factor cascade by losing their phospholipid asymmetry (similarly to platelets) and serve as a procoagulant surface. This role may be important under pathological rather than physiological conditions. In diabetes mellitus and sickle cell crisis phosphatidylserine exposure contributes to thrombotic events [68]. RBCs influence the structure of the fibrin network. Fibrin has larger pores when it is formed in the presence of erythrocytes [69]. Therefore red blood cells are also likely to be an important component of the complex reactions in clot formation and thus determine the ultimate physical properties of fibrin, which affect profoundly the course of its dissolution [70].

CONCLUSION

Platelets and leukocytes are the most important cellular participants in hemostasis, though red blood cells are also likely to play a role. The cellular elements of thrombi have both pro-thrombotic and fibrinolytic properties, they affect each other and interact with components of the blood vessel wall. Their impact on the hemostatic balance is mediated through the structure of the thrombus, release of proteases, inhibitors, structural proteins and cellular lipids. Improved understanding of the cellular contribution to thrombus formation and dissolution would hopefully provide new tools for the treatment of thrombotic disorders.

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ABBREVIATIONS

MMP	Matrix metalloprotease
PAI-1	Plasminogen activator inhibitor 1
PSGL-1	P-selectin glycoprotein ligand-1
RBC	Red blood cell
TF	Tissue factor
TFPI	Tissue-factor pathway inhibitor
t-PA	Tissue-type plasminogen activator
vWf	Von Willebrand factor

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