Thrombosis

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INTRODUCTION

Blood coagulation is initiated by the disturbance of the hemostatic balance following injury, inflammation, endothelial dysfunction, or contact with an artificial surface and involves significant interplay between activation of the enzymatic and cellular processes, adhesion of blood cells, their aggregation, and hemodynamics. Clotting on the inner wall of blood vessels is the major cause of cardiovascular diseases such as myocardial infarction, stroke, and deep vein thrombosis. The formation of homotypic and heterotypic aggregates of activated platelets and neutrophils and their adhesion through selectins, GPIb, and integrin-mediated pathways enhances thrombin generation and subsequent fibrinogen polymerization under coagulating flow conditions. At the same time, cell adhesion and aggregation, typically requiring cell activation, are also influenced by proteins of the blood coagulation cascade.

In addition to platelets, leukocytes and erythrocytes have roles to perform in thrombosis; how they interact with platelets under coagulating flow conditions to stimulate and enhance thrombotic processes has been less well understood. This article seeks to highlight how individual platelets and neutrophils, neutrophil-platelet interactions, and interactions of erythrocytes with these cells activate and promote thrombosis following contact of blood with a defined surface. We intend to provide a fundamental review relevant to biomaterial thrombosis and inflammation–coagulation crosstalk.

COAGULATION BIOLOGY

The Reaction Cascade

Distinct from biomaterial thrombosis, the tissue factor pathway of the coagulation cascade is activated as blood comes in contact with tissue factor following vessel-wall injury. Upon contact with blood, tissue factor forms a complex with endogenous factor VIIa that catalyzes the activation of factor X to Xa. After Xa is formed, it binds factor Va released from platelet stores. The prothrombinase complex (XaVa) on platelets activates prothrombin to thrombin, which cleaves fibrinogen to fibrin monomer. Fibrin monomer polymerizes to form fibrin polymer, which is cross-linked by thrombin-activated factor XIIIa.

The coagulation cascade can also be triggered on the contact of blood with the subendothelium tissues or an artificial surface. This pathway, known as the contact (or intrinsic) pathway of coagulation, involves formation of factor Xa from a series of reactions initiated by factor XII activation on artificial surfaces. Once formed, factor XIIa can activate prekallikrein to kallikrein to amplify factor XIIa formation. Factor XIIa then activates factor XI to XIa, which, in turn, activates factor IX to IXa. Activated platelets participate in the assembly of the intrinsic tenase (IXa/VIIIa) and prothrombinase (Xa/Va) complexes by providing anionic phospholipid binding sites for coagulation proteins. Following an initial production of minuscule amounts of thrombin through the tissue factor pathway, factor XI may be activated to XIa on activated platelets by thrombin to trigger the intrinsic pathway in the absence of the upstream contact pathway proteins, factor XII, prekallikrein, and high molecular weight kininogen.[1] To amplify coagulation, thrombin can activate factor V and factor VIII (to Va and VIIIa), and factor Xa can activate factor VII and factor V (to VIIa and Va). By releasing factor V, which may even be partially active,[2] activated platelets contribute to prothrombinase complex formation for an amplified production of thrombin.

While considerable interplay exists between extrinsic and intrinsic pathways (e.g. TF: VIIa activation of factor IX) and the two pathways are not independent, the concept of TF-dependent and non-TF-dependent initiation of factor Xa production remains an important distinction. Formation of factor Xa is generally viewed as the rate-controlling step in thrombin production.

Platelet Function

Platelet activation, adhesion, and aggregation play a major role in coagulation events. Platelets can be activated by pathological high shear flow (>3000 s^{-1}) or by agonists (e.g., adenosine diphosphate (ADP), thrombin, thromboxane, collagen) released or produced as a result of coagulation reactions or exposed to the blood. The full activation of the platelets involves shape change and release of ADP, as well as activation of glycoprotein

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IIb/IIIa receptors. The aggregation of platelets is mediated through fibrinogen crossbridging of GPIIb/IIIa and von Willebrand Factor (vWF) interactions with GPIbα-IX-V (i.e., GPIb) and GPIIb/IIIa. Once adherent, platelets can recruit other platelets from flowing blood through fibrinogen crossbridging of GPIIb/IIIa and vWF interactions with GP IbIX and GP IIb/IIIa. Fibrinogen binds to GPIIb/IIIa in a specific, saturable manner via the dodecapeptide sequence $\gamma_{400-411}$ on the D domain of fibrinogen. Upon platelet activation, GPIIb/IIIa receptors undergo conformational changes to become competent receptors for soluble fibrinogen and plasma proteins such as vWF. Fibrinogen binds to GP IIb/IIIa receptors (about 50,000 binding sites/platelet) with a dissociation constant (K_d) $\sim$0.1 μM.

The platelet GP IIb/IIIa-fibrinogen interaction is not fully understood and important questions remain concerning the interaction of resting platelets with surface-immobilized fibrinogen. Resting platelets do not appear to bind to adsorbed fibrinogen during short-lived (<50 msec) collisions with fibrinogen-coated beads in a shear flow.[3] However, in whole-blood flow over fibrinogen-coated surfaces, the fibrinogen may be sufficient to capture free-flowing, resting platelets at low shear rates via the $\gamma_{400-411}$ but not Arg-Gly-Asp (RGD) sequences in the carboxy terminus of the α-chain of fibrinogen. These differences may be due to: 1) more favorable cell adhesion mechanics due to wall-directed red blood cell (RBC) particle forces; 2) low-level activation near the wall from RBC-released ADP; 3) differences in contact-area development and force mechanics of doublet formation in suspension flow versus cell-wall interactions; 4) surface-mediated accumulation over several minutes of $\sim$0.1 to 1% of activated platelets in drawn anticoagulated blood; and/or 5) surface-dependent conformational changes of adsorbed fibrinogen. Does GPIIb/IIIa-dependent platelet capture to a growing thrombus require platelet activation? This is a fundamental issue in the dynamics of thrombus growth. The measurement of probabilities of capture, firm arrest, and detachment as a function of interaction time often requires high temporal resolution exceeding that of standard video microscopy. Given the potential for multivalent interaction of polymeric vWF to bind GPIb on resting platelets by accumulating monovalent interactions of micromolar affinity, it also remains unexplained why vWF does not aggregate resting platelets under normal flow. Enhancement of vWF function at various shear rates might be a result of alteration in soluble vWF structure or aggregation state; the GPIb avidity state; and/or the collisional mechanics of doublet formation, a hydrodynamic threshold effect, as in Finger et al.[4] and Lawrence et al.[5] Doggett et al. observed that a critical amount of flow above 70 s^{-1} is needed for platelet GPIb binding to recombinant vWF-A1 domain.[6]

Neutrophil Function

The neutrophils may participate in coagulation by various potential mechanisms. While neutrophils may promote blood coagulation by the deencryption of cell-surface tissue factor[7] or the transfer of tissue factor to platelets through microparticles,[8] they may also release proteases that activate platelets[9] or bind factor X via Mac-1 (CD11b/CD18).[10] Neutrophil elastase may take an active role in the deencryption of tissue factor in the plasma by cleaving tissue-factor pathway inhibitor (TFPI),[11] and this deencrypted tissue factor may become associated on the surface of platelets[12] to promote fibrin formation.

In the baboon femoral arteriovenous shunt Dacron graft model,[13] monoclonal antibody GA6 against P-selectin reduced indium-labeled leukocyte accumulation by 60% over a 2 hr time frame. GA6 Fab2′ fragment reduced leukocyte accumulation by 80% over 2 hr. Anti-P-selectin had no effect on platelet accumulation in the Dacron graft, but dramatically reduced fibrin accumulation by 70%. A marked reduction of fibrin deposition by anti-P-selectin was seen as early as 5 minutes after the initiation of flow. The authors indicated that monocyte and leukocyte expression of tissue factor might be the cause of this increased fibrin deposition. This explanation does not explain the dramatic reduction of fibrin accumulation by anti-P-selectin compared to control at 5 minutes, because expression of tissue factor requires gene activation, transcription, synthesis, and display—a sequence of events that results in elevated tissue factor on monocytes only after 20 minutes. Neutrophils may elevate the production of thrombin at the site of the clot via cross-sectional effects for enhanced platelet capture, tissue factor expression, Mac-1 binding of factor X, the release of proteases such as elastase and cathepsin G, or the display of anionic phospholipid for coagulation pathway assembly. The relative roles of these potential pathways are not fully understood at various flow conditions.

Neutrophil homotypic interactions

Neutrophils present L-selectin on the tips of the microvilli. ICAM-3 and L-selectin are present on resting neutrophils in a competent state for binding. Both L-selectin and ICAM-3 are shed rapidly following neutrophil activation. Upon activation, only about 10% of displayed Mac-1 and LFA-1 is active. LFA-1 (CD11a/CD18) binding to ICAM-3, as well as Mac-1 (CD11b/CD18) binding to an unknown receptor, can facilitate integrin-mediated homoaggregation at low shear rates between 100 and 400 s^{-1}. Above 100 s^{-1}, L-selectin plays an important role in fMLP-stimulated neutrophil homoaggregation. The collision efficiency increases with shear rate to a maximum of ε=0.4 at 400 s^{-1} and then

declines to $\varepsilon=0.15$ at 3000 s^{-1}.[14] This is consistent with observations of CD62P,E,L-mediated rolling displaying a maximum with shear stress, likely due to shear or stress-mediated delivery of microvilli to the rolling contact area[5] or cellular deformation at high flow. Direct imaging of the neutrophil rolling contact area has been achieved[15] to indicate the role of neutrophil membrane deformation and membrane tether formation in regulating adhesion to P-selectin. Similarly, increases in neutrophil membrane rigidity can affect P-selectin-PSGL-1 bond cluster lifetime due to reduction in microvilli extension.[16]

Platelet–neutrophil heterotypic interactions

Recently, the laboratory of Yale Nemerson demonstrated that an inhibitor of tissue factor can greatly reduce blood clotting on purified collagen surfaces lacking tissue factor.[7] This data strongly supports the role of blood-borne tissue factor that is potentially in a cryptic state on neutrophils but is exposed upon activation. Other non-tissue factor mechanisms of neutrophil activation of blood clotting have been described but not prioritized quantitatively. Apoptotic neutrophils or activated neutrophils can display anionic phospholipids, detectable by annexin V binding, which may serve as a cofactor in coagulation factor assembly. Due to the massive size of the neutrophil surface, a few activated neutrophils may be potent catalytic participants in platelet accumulation.[17]

Elastase is known to cause exposure of GPIIb/IIIa on platelets by surface proteolysis.[18] Similarly, cathepsin G can activate platelets. Yamamoto also noted the pro-thrombotic tendency of leukocytes in coagulation assays that was antagonized by the neutrophil protease inhibitor, eglin C.[19] They noted that the procoagulant tendency of activated leukocytes outweighed antiplatelet effects due to NO production or arachidonic acid metabolite exchange with platelets. Neutrophil cathepsin G can also activate factor X bound to Mac-1.[10] The competition and kinetic efficacy of factor X, fibrinogen, and fibrin-binding Mac-1, as well as the prevailing levels of cathepsin G or elastase under flow conditions, remain largely unquantified from a kinetic point of view.

Coagulation and fibrin deposition under flow

De Groot et al.[20] used a calibrated staining assay to measure deposition of peroxidase-labeled fibrin on endothelial cell matrix (containing tissue factor) from heparinized whole blood. They found that fibrinopeptide A generation was not a function of shear rate at 300 or 1300 s^{-1}. Fibrin deposition to the surface was greater at 300 s^{-1} than at 1300 s^{-1}. Fibrin monomer (fibrinogen des-A) concentration was greater in the perfusion fluid at 1300 s^{-1}. In this study, the perfused blood was anti-coagulated with heparin, which may emphasize the role of fibrin-bound thrombin that is resistant to heparin-mediated inhibition. The impaired fibrin polymerization at high shear was suggested to be due to increased removal of the monomer from the surface, but the physical hydrodynamics of this are poorly understood. This study is in contrast to a study with nonanticoagulated whole blood perfused over partially denuded rabbit aortas where platelet and fibrin deposition both increased with shear rates up to 1500 s^{-1}.[21] Fibrin deposition and its shear dependency are highly sensitive to the presence or absence of cellular contributions and prevailing anticoagulation conditions. Neutrophil adhesion to preformed endothelial matrix and fibrin under shear flow was studied recently by Kuijper et al.[22] Although neutrophils can adhere to fibrin via Mac-1 (CD11b/CD18) at shear stresses up to 20 dynes/cm^2, soluble fibrinogen inhibited the interaction. Fibrin-mediated steric hindrance of neutrophil attachment to TNF-α–treated endothelium was noted by Kirchhofer et al.[23] As a bulky structure, it is possible that fibrin can prevent selectin interactions with endothelium that would normally facilitate rolling and subsequent firm adhesion.

A potential role for red-blood-cell adhesion in thrombosis

Palabrica et al.[13] noted that anti-P-selectin appeared to reduce, through an unknown mechanism, the content of red blood cells on a Dacron-graft thrombus as indicated by scanning electron microscopy of the clot surfaces. This observation raises the issue of RBCs binding to clots or activated leukocytes. The RBCs help push platelets to the reactive wall, as well as leak ADP under high-shear conditions. While numerous studies have looked at thrombospondin and CD36-dependent red-blood-cell adhesive dynamics during sickle cell anemia, malaria, and diabetes, little is known about the adhesive interaction of normal RBC during thrombosis. Treatment of healthy RBC with 2 μM a23817 and 0.5 mM $CaCl_2$ causes the loss of lipid asymmetry in up to 40% of the RBC as indicated by phosphatidylserine (PS) exposure detectable by FITC-annexin V binding.[24] In their work, CD36 was not detected on ionophore-treated normal RBC. PS exposure by RBC is sufficient for expression of procoagulant activity, as well as alternative complement pathway activation as indicated by C3b deposition.[25] It has been shown that PS on RBC was sufficient for the binding of RBC to endothelial matrix or purified thrombospondin. Interestingly, even normal RBC could bind immobilized thrombospondin at a level ~ten-fold greater than RBC binding to albumin. This suggests that RBC-binding thrombospondin may be important in the adhesion of normal RBC to activated spread platelets that release thrombospondin and display CD36. Platelet-released

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thrombospondin is an important mediator of platelet aggregation via a CD36-dependent mechanism. A role for platelet–RBC interaction in sickle cell disease has been reported to be partially mediated by thrombospondin.[26] Platelet-RBCs are routinely observed (and ignored) in flow cytometry of anticoagulated whole blood. RBC captured in the early thrombus may display PS due to alternative complement pathway activation, and thus become procoagulant and adhesive for platelet thrombospondin. These mechanisms may be especially operative at low-shear rates if binding is extremely weak and transient.

Complement System/Activation

The complement pathway also plays a role in biomaterial function, thrombosis, and biomaterial infections. Bacterial killing in coagulating blood involves numerous interactions among coagulation factors, platelets, neutrophils, bacteria, and the complement system. Infections in coagulated blood present an important class of clinical complications in the context of artificial biomaterials, infective endocarditis, mechanical and bioprosthetic heart valve endocarditis, and wound healing. Yet, little is known about the role and dynamics of various biochemical or biological participants in regulating bacterial survival during blood coagulation. Through the classical or alternative pathway, complement-based lysis of bacteria depends on the generation of C3b and C5b on bacterial surfaces, thus triggering the formation of the membrane attack complex (C5b-9). The classical pathway is calcium and magnesium-dependent, while the alternative pathway is magnesium-dependent. Gram-negative bacteria such as *Escherichia coli* are potent activators of the alternative pathway. Also, C3b and iC3b act as opsonins; C3b binds neutrophil complement receptor CR1, while iC3b (but not C3b) binds neutrophil CR3 (Mac-1) and CR4.

Early work on platelet activation and aggregation in the presence of complement has demonstrated that thrombin can act on the platelet surface as a low-activity C3 convertase and high-activity C5 convertase. In solution, thrombin cleaves C3 (presumably at the Arg_{69}–Ala_{70} bond) to produce fragments roughly the size of C3a and C3b that have no anaphylatoxin or hemolytic activity, respectively. Thrombin can very rapidly cleave C5 to a C5b-like fragment and a C5a-like fragment that lacks chemotactic activity unless further digested by thrombin for 16 hr. In fact, levels of anaphylatoxin antigens C3a, C4a, and C5a are ten-, four-, and 60-fold higher, respectively, in freshly prepared serum (from whole blood maintained for 30 min at room temperature in glass tubes) than in EDTA-treated plasma, demonstrating that coagulation can activate complement. However, this level of generation of C3a, C4a, and C5a in serum represents only a ~3 to 10% conversion of C3, C4, and C5. Also, thrombin can cleave C9 to C9a and C9b, which remain associated without loss of hemolytic function.

BIOMATERIAL THROMBOSIS

Fibrin Formation by Adherent Neutrophils

Implantation of biomaterials is generally followed by adsorption of plasma proteins such as albumin, fibrinogen, vWF, and vitronectin onto the artificial surface. Neutrophils, via Mac-1 (CD11b/CD18), can adhere to immobilized fibrinogen. The regional neutrophil density can increase by an inflammatory response that may even be triggered by biomaterial-adsorbed fibrinogen.[27] Once adherent, neutrophils can mediate the capture of flowing neutrophils near the surface via a L-selectin-PSGL-1 (P-selectin glycoprotein ligand 1) mechanism.

Our studies have shown that under venous flow conditions, individual surface adherent neutrophils can promote fibrin deposition in the presence of the intrinsic coagulation protein factor XIIa.[28] On the perfusion of platelet-free plasma (PFP) over fibrinogen-adherent neutrophils at a wall shear rate (γ_w) of 62.5 s^{-1}, dense fibrin deposition in close proximity to the neutrophils, especially downstream of each cell, was observed (Fig. 1A). The time required for this fibrin deposition on

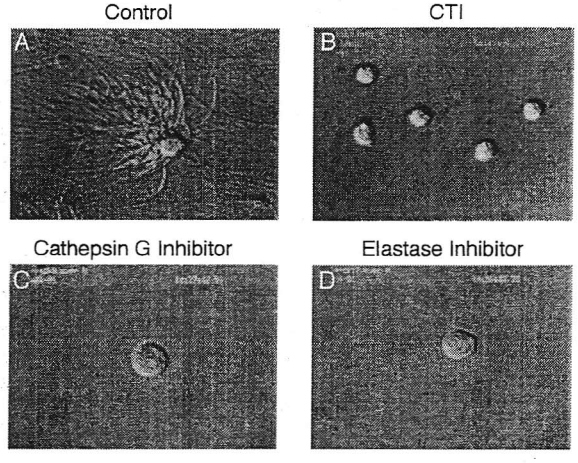

Fig. 1 XIIa-dependent fibrin formation around neutrophils. As recalcified citrated PFP was perfused over surface-adherent neutrophils at a shear rate of 62.5 s^{-1} for 20 min, dense fibrin deposition was observed around each neutrophil, especially downstream of each cell (A). No fibrin was formed even at $t=35$ min in the presence of CTI (B). Fibrin accumulation was attenuated when either cathepsin G inhibitor (C) or elastase inhibitor (D) was present in the PFP being perfused over neutrophils (also treated with the matching inhibitor). Flow is from right to left.

neutrophils was prolonged by 40 to 50 min by inhibition of factor XIIa with corn tryprin inhibitor (CTI), demonstrating the role of contact pathway in this fibrin accumulation around neutrophils. Fig. 1B illustrates no fibrin formation at $t=35$ min in the presence of CTI.

One of the mechanisms through which individual fibrinogen-adherent neutrophils promote fibrin deposition is capture of short fibrin protofibrils flowing in recalcified plasma in contact with in-vitro surfaces. This mechanism may involve Mac-1 (CD11b/CD18)–mediated capture and, to a much lesser extent, capture by nonspecific cross-sectional capture effects that is analogous to particle capture by a sphere under flow. However, fibrin capture is not the sole process responsible for fibrin deposition. In addition to blockade of Mac-1 and inhibition of XIIa, the inhibition of either one of the two neutrophil proteases, cathepsin G and elastase (Fig. 1C and 1D), also attenuated fibrin formation around adherent neutrophils.[28] This demonstrated that fibrinogen-adherent neutrophils can promote FXIIa-dependent thrombin generation and subsequent fibrin formation in a platelet-independent manner through pathways mediated by the released proteases elastase and cathepsin G.

The reduction in fibrin formation by either the blockade of Mac-1, inhibition of XIIa, or inhibition of either one of two proteases, cathepsin G and elastase, may be attributed to the suppression of prothrombinase formation (factor Xa and factor Va) on the neutrophil surface (Fig. 2). Because cathepsin G has been found to activate Mac-1–bound factor X,[10] decline in factor Xa levels can be expected by inhibition of cathepsin G or the blockade of Mac-1. Moreover, inhibition of elastase (or cathepsin G) may suppress factor V activation by these proteases,[29] resulting in reduced levels of factor Va. Inhibition of factor XIIa would reduce the formation of the prothrombinase complex (XaVa) by reducing XIa and consequently IXa and then Xa production.

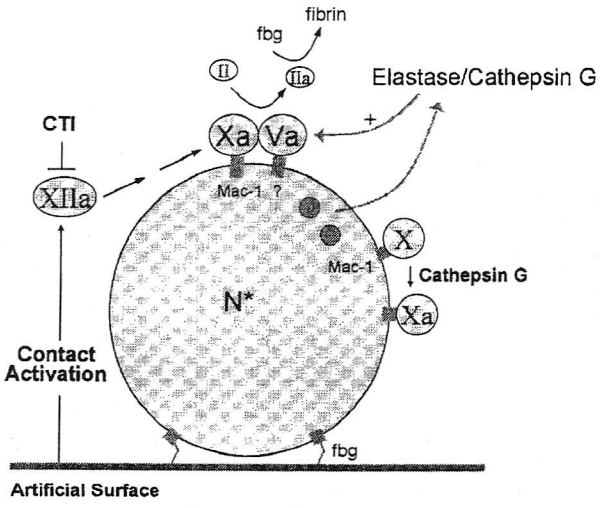

Fig. 2 Procoagulant activity on the neutrophil surface. Individual neutrophils may enhance coagulation in a platelet-independent manner through prothrombinase formation on the neutrophil surface. Abbreviations: fbg, fibrinogen; CTI, corn trypsin inhibitor; XIIa, factor XIIa; X, factor X; Xa, factor Xa; Va, factor Va; II, prothrombin; IIa, thrombin; N*, activated neutrophil.

While blockage of Mac-1 attenuated fibrin formation on neutrophils, it is difficult to distinguish fully the role of Mac-1 on fibrin capture versus its role in thrombin formation, because Mac-1 binds fibrin(ogen) and factor X to the neutrophil.[10] Since neutrophils can bind high molecular-weight kininogen (which can bind prekallikrein and factor XI), as well as factor XII,[30] antibodies against CD11b/CD18 will clearly interfere with fibrin binding but may also attenuate pathways leading to Xa and thrombin formation on the neutrophil surface. Also, factor XIIa is a known activator of neutrophils, and CTI would be expected to attenuate kallikrein-mediated release of elastase by neutrophils exposed to recalcified plasma.[31]

In the absence of platelet deposition and red-cell motion, fibrin accumulation over neutrophils in the presence of contact activation was inversely correlated with wall shear rate.[28] The density of fibrin deposited around each neutrophil at a wall shear rate of 250 s^{-1} was significantly lower than that at a wall shear rate of 62.5 s^{-1}. Under the conditions present in these experiments, an increase in flow rate may decrease the time available for interaction between the Mac-1 receptors and the complementary fibrin domain or enhance the off-rate of binding. Alternatively, dilution effects of key factors (e.g., Va, Xa, or thrombin) may be enhanced at high flow. Under physiological conditions, the presence of red cells and their motion would considerably influence coagulation. Their presence would increase the collision frequency of cells with the wall to enhance platelet aggregation and platelet deposition, respectively. Additionally, rouleaux formation would support vessel-wall adhesion of neutrophils through their outward displacement under flow.[32]

Neutrophil Enhancement of Platelet-Dependent Coagulation

Platelets can adhere to plasma proteins immobilized on biomaterials through the same mechanisms that are involved in their adhesion to the vessel wall. However, unlike collagen, fibrinogen provides a surface onto which platelets can spread, but do not transform into fully procoagulant structures on their own. Our results have shown that adherent neutrophils can interact with fibrinogen-adherent platelets to turn them into activated procoagulant structures.[28] As platelet-free plasma was

perfused over a neutrophil-coated surface, a platelet-coated surface, and a surface coated with both neutrophils and platelets, dense fibrin deposition was observed only on the latter surface suggesting that neutrophil-platelet aggregation and adhesion can have profound roles in promoting coagulation under venous flow conditions even in the presence of factor XIIa–inhibition by CTI. This fibrin formation was reduced by specific peptide inhibitors against elastase and cathepsin G, indicating that the interaction of neutrophils with platelets to facilitate fibrin formation is mediated through these neutrophil proteases.

As we compared fibrin formation on fibrinogen-adherent neutrophil–platelet cell mixtures to that on collagen-adherent platelets (no neutrophils), we found the amounts of fibrin deposited to be equivalent (Fig. 3); neutrophils on a fibrinogen-coated surface had enhanced platelet-dependent fibrin formation to the levels supported by platelets alone on a collagen surface. Because fibrinogen-adherent platelets, unlike collagen-adherent platelets, are not fully activated procoagulant structures, these observations demonstrated that neutrophils, even in the absence of collagen, can activate platelets on their own and subsequently turn them into fully coagulating structures.

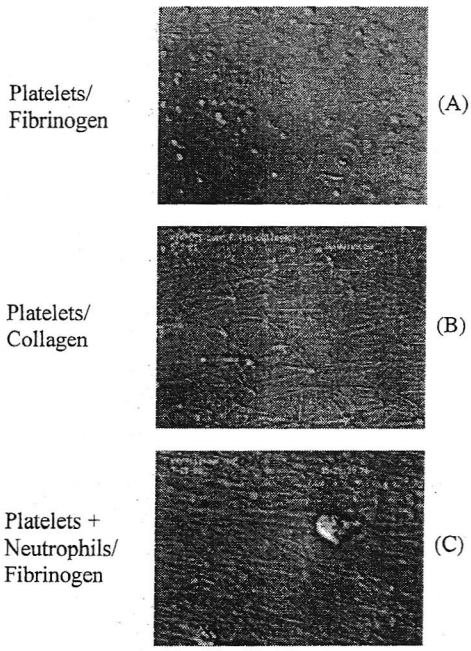

Fig. 3 Neutrophil promotion of fibrin formation in a platelet-dependent manner. On the perfusion of PFP at a shear rate of 62.5 s^{-1} for 25 min, while collagen-adherent platelets (B) supported more fibrin formation than fibrinogen-adherent platelets (A), neutrophils enhanced fibrin formation on fibrinogen-coated platelets (C) to levels supported by collagen-adherent platelets.

Table 1 Effects of cathepsin G and elastase on thrombin generation

Environment		Burst time (t_{50}), min[a]
PRP, CTI	No protease	53±1
	Cathepsin G (100 μM)	18±1
	Elastase (100 μM)	53±19
	Cathepsin G+Elastase	17±1
PFP, CTI	Cathepsin G+Elastase	>120

PRP=platelet-rich plasma, PFP=platelet-free plasma; CTI (corn trypsin inhibitor) to inhibit factor XIIa from the contact system.
[a]Data shown represent the means ±S.D. of the observations.

Rather than being localized on and near neutrophils, the formed fibrin deposited uniformly over the entire platelet surface of the flow chamber. This observation suggests that neutrophil-promoted fibrin formation on platelets is a consequence of some intercellular signaling event and/or catalysis of coagulation biochemistry between neutrophils and platelets that is mediated by elastase/cathepsin G. Several pathways exist by which neutrophils may trigger thrombin production and subsequent fibrin formation in a platelet-dependent manner, including: 1) elastase[33] or cathepsin G[9] enhancement of activation of spread platelets; or 2) elastase/cathepsin G cleavage of plasma zymogens such as factor V[29] and/or X.[10] While mechanisms of P-selectin–mediated adhesion under flow are eliminated in these studies, P-selectin–dependent platelet-neutrophil signaling did not appear to play a role in the process. We have also found that among the two neutrophil proteases, cathepsin G has a more potent role in enhancing platelet dependent coagulation.[34] A coupled activity by both cathepsin G and elastase is not a prerequisite to achieve full activation of fibrinogen-adherent platelets; neutrophil cathepsin G alone can fully activate platelet-dependent coagulation. Moreover, platelet activation is the dominant mechanism through which cathepsin G turns fibrinogen-adherent platelets procoagulant. Using fluorimeter assay, the burst time (t_{50}, the time to reach 50% conversion of a fluorogenic thrombin substrate) for thrombin generation was measured to quantify the effect of elastase and cathepsin G on coagulation.[34] While the addition of cathepsin G to PRP reduced the peak thrombin generation from 53 min to 18 min, the addition of elastase did not make a difference (Table 1).

The addition of phosphatidylserine/phosphatidylcholine vesicles (PSPC) alone to CTI-treated platelet-free plasma produced little thrombin, indicating that mere phosphotidylserine exposure during platelet activation is not sufficient for coagulation of the plasma.[34] When neutrophil elastase was added to PFP containing PSPC, thrombin generation was also minimal. However, a

detectable level of slow thrombin production was observed when cathepsin G or elastase/cathepsin G was added to PFP/CTI containing PSPC. These observations demonstrated that the presence of a negatively charged phospholipid surface is not sufficient to initiate coagulation. Cathepsin G is cationic and the slight increase in thrombin levels on addition of PSPC supports the view that cathepsin G–mediated cleavage of coagulation factors on a negatively charged surface may help support, in a secondary role, cathepsin G–mediated platelet activation. In comparison to cathepsin G–enhanced thrombin generation in PFP containing PSPC, a five times higher thrombin production in PRP (presence of platelets) indicated that a platelet has additional means, beyond merely presenting negative phospholipid, through which it responds to cathepsin G stimulation. These results were in agreement with studies by Sumner et al.,[35] which show that PS exposure does not correlate with factor Xa or thrombin production on platelets and suggest that surface participants other than PS are involved in coagulation.[36] Whereas display or release of active tissue factor by neutrophils[7,8] to induce coagulation is possible, tissue factor did not appear to have a role in this process.[28,34]

Adhesion of Normal Erythrocytes

While it is known that sickle and infected erythrocytes can directly adhere to the endothelium, a common assumption is that normal red blood cells are passive during coagulation events and become entrapped in fibrin. We have examined capture and adhesion of normal erythrocytes to surface-adherent neutrophils and platelets under low-flow conditions as a mechanism of RBC accumulation distinct from passive entrapment within fibrin fibers.[37] As washed erythrocytes were perfused over collagen-adherent platelets at a wall shear rate (γ_w) of 50 s^{-1} for 5 min to investigate the interactions between erythrocytes and platelets under flow, many events of red cell capture, transient tethering, and/or firm adhesion (Fig. 4A) to platelets were observed. To examine the dependence of erythrocyte adhesion on platelet activation, erythrocyte adhesion on collagen-adherent platelets was compared to that over fibrinogen-adherent platelets. The density of red cells captured was four-fold higher on collagen-adherent platelets, demonstrating that red-cell adhesion to platelets at depressed venous flow conditions is regulated by the platelet activation state. Moreover, red-cell adhesion to fibrinogen-adherent platelets that had been directly stimulated with the GPVI agonist convulxin was 2.7-fold higher than to unstimulated fibrinogen-adherent platelets.[37] This verified the role of platelet activation in mediating these red-cell adhesion events.

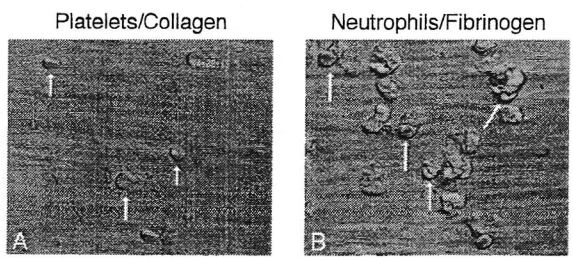

Fig. 4 Erythrocyte capture by activated platelets and neutrophils. As washed red cells were perfused over collagen-adherent platelets (A) or fibrinogen-adherent fMLP-treated neutrophils (B) at a wall shear rate of 50 s^{-1}, firm adhesion of red cells (arrows) was observed.

Experiments in which potential mediators of these interactions, such as P-selectin, CD36, GPIb, GP IIb/IIIa, TSP, or vWF, were blocked by antibodies demonstrated partial roles for CD36 and GPIb in adhesion of red cells to activated platelets.[37] When fibrinogen (3 mg/ml) was added to the red cells prior to their perfusion over platelets, cell adhesion was significantly blocked. This demonstrated that at physiological concentrations, fibrinogen plays a protective role in preventing red-cell adhesion to collagen-activated platelets. Red-cell adhesion to platelets was Ca^{2+}- or Mg^{2+}-dependent.

When washed red cells were perfused over fibrinogen-adherent neutrophils at a wall shear rate of 50 s^{-1} for 5 min, a few transient adhesion events were observed between red cells and fibrinogen-adherent neutrophils. However, when neutrophils were activated with N-formyl-Met-Leu-Phe (fMLP), the number of pausing events and firmly adherent red cells (Fig. 4B) to these activated neutrophils dramatically increased. fMLP caused a sixfold increase in RBC adhesion to neutrophils. Erythrocyte adhesion to fMLP-activated neutrophils at depressed venous flows, unlike RBC adhesion to collagen-activated platelets, was not blocked by soluble fibrinogen. Adhesive interactions between erythrocytes and activated neutrophils were found to be mediated by Mac-1 (CD11b/CD18). One of the receptors on the red cell was detected to be ICAM-4 (LW blood group). Blocking antibodies against CD36 or TSP did not show any effect. Consistent with β_2-integrin function, the adhesion of erythrocytes to activated neutrophils was markedly inhibited by EDTA.

From adhesion assays conducted at $\gamma_w = 25$, 50, 75 and 100 s^{-1}, we observed that the number of adherent red cells to collagen-adherent platelets decreased as the shear rate increased.[37] These observations indicate that the RBC adhesion to platelets is significant only at depressed venous flow conditions (below 100 s^{-1}). Similarly, RBC adhesion to fMLP-treated neutrophils was inversely correlated to shear rate, and it was significantly reduced at γ_w above 100 s^{-1}.

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While it is known that vascular compression devices help prevent deep vein thrombosis (DVT), the molecular mechanisms by which they do so have been unclear. Our observation that firm adhesion of red cells to platelets and neutrophils is efficient at shear rates below 100 s^{-1} may explain this phenomenon. Reduced shear rates can allow red-cell adhesion to the cells adherent to inflammed venular endothelium causing occlusion and pain. Vascular compressions may lead to a slight increase in blood movement and wall shear rate and shear stress, which, as explained previously, may impede erythrocyte capture and/or reverse adhesion and subsequent vasoocclusion, thus providing DVT prophylaxis to the patient.

CONCLUSION

Adherent neutrophils, being more concentrated at sites of inflammation, have a considerable impact on biomaterial thrombosis. Fibrinogen-adherent neutrophils are sufficient to initiate and amplify coagulation on a biomaterial surface in the presence of activated XIIa. Additionally, the effects of neutrophil-platelet interactions, which primarily occur during adhesion and heterotypic aggregation, on subsequent fibrin formation have been reviewed in a system that decouples flow-regulated adhesion events from flow-regulated coagulation biochemistry. Inflammatory processes, causing neutrophil or platelet arrest and activation on an activated endothelium or a biomaterial surface, can be followed by receptor-mediated capture of red blood cells under low flow conditions. In addition to biomaterial thrombosis, these events may be clinically relevant in situations devoid of collagen or tissue factor such as those involving endothelial dysfunction and neutrophil activation during deep vein thrombosis.

ARTICLES OF FURTHER INTEREST

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