Platelets in Inflammation and Thrombosis
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Abstract—For many years it has been known that platelets play an important role in thrombosis and hemostasis. In recent times, however, it has become evident that platelets also have relevant functions in inflammation. It was shown that thrombosis and inflammation share several key molecular mechanisms and in fact are 2 intrinsically linked processes. In this review, we intend to give a short overview with emphasis on work stemming from our laboratory. (Arterioscler Thromb Vasc Biol. 2003;23:2131-2137.)

Key Words: platelets • inflammation • thrombosis • atherosclerosis • hemostasis

Endothelial Response to Injury and Platelet Adhesion
One linkage between thrombosis and inflammation can be seen at the molecular and cellular levels in the endothelium. The primary molecules responsible for initiation of platelet and leukocyte adhesion, von Willebrand factor (VWF) and P-selectin, respectively, are stored in the same storage granules called Weibel-Palade bodies.1–4 VWF is essential for Weibel-Palade body formation,5 and therefore mice with VWF deficiency cannot store P-selectin in endothelium, leading to its partial degradation. This makes the mice less responsive to inflammatory stimuli.6 On cellular activation with either prothrombotic secretagogues, such as thrombin, or proinflammatory secretagogues, such as histamine, Weibel-Palade bodies fuse with the cell membrane and expose membrane-bound P-selectin and soluble VWF at the cell surface.7

Fairly recently it was found that both VWF and P-selectin, freshly secreted from activated endothelium, mediate platelet interaction with the endothelial surface. As to which of these 2 molecules will be the primary player seems to depend on the shear rate of the particular vessel. André et al8 showed that in stimulated veins with low shear rates, large numbers of resting platelets translocate (a stop-and-go phenomenon) on the shear rate of the particular vessel. Andreé et al 8 showed that in stimulated veins with low shear rates, large numbers of resting platelets translocate (a stop-and-go phenomenon) on VWF that is transiently bound to the endothelial surface. Under these conditions, platelet glycoprotein (GP) GPIbα mediates platelet adhesion. This transient adhesion process is likely terminated by the cleavage of VWF multimers by ADAMTS13,9 the enzyme deficient in patients with inherited thrombotic thrombocytopenic purpura.10 At higher venular shear rates, activated and nonactivated platelets are able to roll on activated endothelial cells (similar to the well-known mechanism of leukocyte rolling), a process mediated by P- and E-selectin.11,12 P-selectin glycoprotein ligand-1 (PSGL-1), the main ligand of P-selectin, expressed on leukocytes was also found on platelets and was shown to be at least partially responsible for this phenomenon.13 GPIbα is another ligand of P-selectin and may therefore also support platelet rolling on activated endothelium.14 P-selectin–dependent interaction of platelets with the vessel wall was demonstrated during ischemia and reperfusion.15

Platelets in Thrombosis and Hemostasis
A severe vascular injury will lead to deendothelialization and exposure of subendothelium. VWF, previously produced and released by the endothelium or adsorbed from plasma onto exposed tissue, is crucial for platelet adhesion, in particular in vessels with high shear rates, such as arteries and arterioles.16–18 Here also VWF binds to its platelet membrane receptor, GPIb-IX-V, establishing a transient bond that slows down platelets and thus facilitates their activation.19 Absence of VWF, as investigated in VWF-deficient mice by intravital microscopy, significantly delays platelet deposition on a ferric chloride–injured vessel wall and significantly increases the time required for the formation of a first thrombus.20,21 In humans, absence of the GPIb-IX-V complex results in a severe bleeding disorder known as Bernard Soulier syndrome, which, besides impaired VWF binding, is characterized by thrombocytopenia and giant platelets.22

After tethering, rapid conversion to stable platelet adhesion is required to promote thrombus formation. This process is primarily mediated by the interaction of platelet surface–expressed integrins such as integrin α5β1, and the immunoglobulin-like receptor GPVI with collagen. These interactions promote platelet activation and as a consequence promote a shift to a high-affinity state of the major platelet integrin αIIbβ3, which mediates platelet aggregation. Collagen binding also induces the release of adenosine diphosphate and thromboxane A2, platelet agonists that additionally activate adherent platelets in an autocrine manner by amplifying
integrin adhesiveness and thereby promote thrombus growth. The serine protease thrombin generated by the coagulation cascade is another important platelet agonist. Besides activating platelets through cleavage of its G-protein–linked protease-activated receptors, thrombin can also cleave GPV of the GPIb-IX-V complex, which allows thrombin binding to GPIbα, a process that also results in platelet activation. Absence of GPV on platelets, as tested in GPV-null mice, results in enhanced platelet responsiveness to thrombin and in faster thrombus growth in vivo.

The absence of αmβ3 integrin in human causes a severe bleeding disorder called Glanzmann thrombasthenia. Observation of injured arterioles of β3-deficient mice showed defects in stable platelet adhesion and, even more importantly, complete absence of aggregate formation (Hynes and Wagner, unpublished observations, 2002). Thus, β3 integrin is absolutely crucial for platelet crosslinking. Fibrinogen was generally considered the main ligand crosslinking the αmβ3 integrins on neighboring platelets. Surprisingly, in the absence of fibrinogen, as tested in fibrinogen-deficient mice (Fg−/−), thrombi still form readily. Thrombi of these mice, however, are unstable and fail to resist shear stress, which results in frequent embolization of the entire thrombus, with vessel occlusion downstream of the injured area. Clearly, fibrinogen/fibrin is required to secure thrombus stability. In addition to the important function of the fibrin meshwork, which ensures thrombus anchoring at the site of injury, we recently found that thrombi are stabilized by the interaction of the C-terminal γ chain of fibrinogen/fibrin with platelet αmβ3 integrin, an interaction that has been demonstrated in vitro. Like Fg−/− mice, mice deficient in the last 5 amino acids of the fibrinogen γ chain (FgγΔ5 mice) showed enhanced embolization on vascular injury. However, in contrast to the Fg−/− mice, the FgγΔ5 mice were able to form occlusive thrombi at the site of injury because fibrin can still form in these animals.

The very rapid thrombus growth in the absence of fibrinogen questions the key role of fibrinogen in the formation of platelet-platelet aggregates in vivo. The ligand responsible for the fast growing thrombi in the fibrinogen-deficient mice was not VWF. Mice deficient in both VWF and fibrinogen were still capable of forming thrombi. Platelets of fibrinogen-deficient mice as well as of FgγΔ5 mice accumulate excessive amounts of fibronectin, indicating that fibronectin competes with fibrinogen for binding to αmβ3, which mediates internalization of these molecules into platelet α-granules. Fibronectin is known to support platelet adhesion and spreading. However, the role of fibronectin in thrombus formation and stabilization was not well defined. We therefore tested in a conditional fibronectin knockout mouse model whether fibronectin is an important ligand in thrombus growth. Deficiency of plasma fibronectin did not affect the initial platelet adhesion, but it resulted in a delay of several minutes in thrombus formation. Thrombi of fibronectin-deficient mice continuously shed platelets or small platelet clumps, leading to significantly slower thrombus growth than seen in wild-type mice. Therefore, fibronectin is an important ligand mediating platelet-platelet interactions within growing thrombi.

Thus, from the work with transgenic mice and previous in vitro work under flow, a clearer model of thrombus growth in high shear rate conditions emerges. VWF mediates the early platelet adhesion to the injured site. Fibronectin rapidly cross-links platelets by binding to activated αmβ3 integrins. Fibrin is generated and anchors the growing thrombus to the site of injury. Platelets attach to the fibrin/fibrinogen meshwork also by αmβ3 integrin, which stabilizes the thrombus and slows down its growth. Finally, at very high shear rates, near vessel occlusion, VWF is required again because thrombus growth in arterioles of VWF-deficient mice tends to arrest before vessel occlusion, with high-shear channels remaining open. Although this has not been demonstrated in vivo, VWF likely fortifies the thrombi by binding to GPIbα, in addition to the αmβ3-mediated linkages.

In recent work in collaboration with David Phillips, we were able to identify another important new ligand for αmβ3, CD40L. This molecule represents yet another link between inflammation and thrombosis. CD40L, a transmembrane protein of the tumor necrosis factor (TNF) family, is expressed not only on cells of the immune system but also on activated platelets. It is stored in platelets and gets externalized and shed on platelet activation. CD40L-deficient mice exhibited delayed vessel occlusion and frequent embolization, a phenotype that could be rescued by the injection of recombinant soluble CD40L. Binding to the β3 integrin through its KGD amino acid sequence, may directly activate the ligand-binding activity of αmβ3, as reported for RGD peptides. Because of its small size, it is less likely that CD40L directly cross-links platelets like other αmβ3 ligands.

Because fibrin formation by thrombin is very important for thrombus stability, we will discuss a newly understood role of P-selectin, the adhesion receptor for leukocytes, in this process. Two observations first pointed to a role of P-selectin in coagulation. First, Palabrica et al. showed that leukocytes are recruited into growing thrombi and promote fibrin deposition that is inhibited by antibodies to P-selectin. Second, Hartwell et al. generated a knock-in mouse of P-selectin that lacks the cytoplasmic domain (ΔCT mouse) and is characterized by high levels of soluble P-selectin in plasma. Blood of these mice clotted (formed fibrin) faster, indicating that the shedding of P-selectin generated a procoagulant state in the animals. Furthermore, excessive fibrin deposition on platelet thrombi in ex vivo flow chamber studies was observed. In vivo, the hemorrhage produced by a local Schwarzmann reaction was significantly smaller in ΔCT than in wild-type mice because of a protective layer of fibrin deposited rapidly on the inside of the vessel wall. This effect could be reproduced by infusion of a chimeric P-selectin immunoglobulin fusion protein (P-sel-Ig) into wild-type mice, as shown in Figure 1.

Celi et al. showed that P-selectin, either expressed on activated cells or purified, upregulates tissue factor (TF) expression on monocytes in vitro, and more recently TF was found to circulate in blood as part of microparticles. We showed that P-sel-Ig induced leukocytes to form such TF-containing microparticles and that these microparticles were responsible for the procoagulant activity in the ΔCT mice.
Higher than normal levels of soluble P-selectin in blood were found in thrombotic consumptive platelet disorders, such as disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, or heparin-induced thrombocytopenia, disorders that are all associated with thrombotic and thromboembolic complications. Furthermore, higher levels of soluble P-selectin are predictive of future cardiovascular events. These findings support the concept of P-selectin having procoagulant activity.

Hrachovinova et al recently found that P-sel-Ig can induce the formation of TF-containing microparticles in human as well as in mouse blood and that this is mediated through binding to PSGL-1 on leukocytes. Compared with wild-type mice, mice that were deficient in PSGL-1 (PSGL-1−/− mice) produced fewer microparticles both spontaneously in old age as well as after P-sel-Ig infusion. Thus, by inducing the generation of soluble TF, P-selectin and its receptor PSGL-1 play an important role in hemostasis.

Because TF-containing microparticles were generated from leukocytes, their adhesion interactions are similar to those of the cell of origin. It was shown by intravitral microscopy that native microparticles might be directly involved in thrombin generation because they specifically bind to growing thrombi at sites of vascular injury. In vitro, this was shown to be mediated by P-selectin and a carbohydrate ligand with some involvement of TF. When infused into mice, in vitro-produced microparticles from monocytes were also found to be recruited to thrombi in a P-selectin/PSGL-1–dependent manner.

These observations have therapeutic implications. Inhibition of P-selectin/PSGL-1 interaction would reduce procoagulant activity. This is likely why recombinant soluble PSGL-1 promotes thrombus dissolution and why P-selectin–deficient mice have a slightly prolonged bleeding time. On the other hand, increasing P-selectin/PSGL-1 interactions would have an opposite effect. This too may be desirable in certain clinical situations. In a mouse model of hemophilia A, infusion of P-sel-Ig produced a 20-fold increase in TF-containing microparticles, which significantly facilitated fibrin formation and normalized the impaired tail-bleeding time of these mice within 6 hours. P-sel-Ig could become a new method to treat hemophilia patients, in particular those producing alloantibodies to factor VIII or IX.

**Platelets in Inflammation**

Inflammation leads to an imbalance between procoagulant and anticoagulant properties of the endothelium that can lead to a local stimulation of the coagulation cascade. TNF-α, the first proinflammatory cytokine released at the site of infection, is a potent inducer of the immune defense mechanism and a mediator of leukocyte recruitment. It promotes a procoagulant state by inhibiting synthesis of the anticoagulant protein C and by eliciting TF production on the endothelium and monocytes, thereby stimulating thrombin and fibrin formation. TNF-α has pleiotropic effects, like most cytokines. Nevertheless, it was rather surprising that TNF-α, at concentrations found in lethal or sublethal sepsis, strongly inhibited thrombus growth in the ferric chloride vascular injury model and prolonged bleeding time of treated mice (B. Cambien, unpublished data, 2003). This transient (lasting less than 2 hours) powerful antithrombotic effect of TNF-α is not mediated by a platelet TNF receptor but rather through the stimulation of inducible NO synthase by TNF receptors in the vessel wall with consequent release of NO, a strong inhibitor of platelet activation. Thus, perhaps in the early phases of infection, when leukocyte recruitment is of primary importance to prevent bacterial spread, the formation of thrombi obstructing blood flow is not desired. In addition, NO produced constitutively by endothelial cells significantly inhibits Weibel-Palade body release, thus reducing platelet adhesion to endothelium.

Inflammation is also characterized by a multitude of interactions between leukocytes, endothelial cells, and platelets. Irrespective of its etiology, inflammation causes endothelial activation. Activated endothelial cells express cell adhesion molecules such as P- and E-selectin, which mediate leukocyte rolling, the first step in the cell-adhesion cascade. Rolling leukocytes become activated by chemokines such as monocyte chemoattractant protein 1 and RANTES, presented by the activated endothelial surface. The activated leukocytes can bind to other endothelial adhesion molecules, such as intercellular adhesion molecule-1 and vascular adhesion molecule-1, and start transmigrating into the vascular intima. Targeted disruption of the P- and E-selectin gene in the mouse results in marked inhibition of leukocyte rolling and delayed recruitment of monocytes into sites of inflammation and in enhanced susceptibility to infection.
Atherosclerosis is a typical example of a chronic inflammatory process. Absence of P-selectin was shown to reduce and delay atherosclerotic lesion formation in atherosclerosis-prone LDLR-deficient or apoE-deficient mice. More recently, in a bone marrow transplant animal experiment, we demonstrated that not only endothelial P-selectin but also platelet P-selectin contributes to atherosclerotic lesion formation in the apoE-deficient mice, demonstrating direct platelet and platelet P-selectin involvement in the atherosclerotic process. Interestingly, P-selectin also influences lesion maturation in a dose-dependent manner (Figure 2). P-selectin mediates rosetting of monocytes and neutrophils with circulating activated platelets, an interaction that increases monocyte adhesion to endothelial cells and may finally facilitate macrophage accumulation in the vessel wall. Platelets adherent to subendothelial matrix also support leukocyte rolling, adhesion, and transmigration through the interaction of their P-selectin with leukocyte PSGL-1. The process of leukocyte rolling allows platelet activating factor to stimulate leukocyte integrin Mac-1, become activated, and mediate firm leukocyte adhesion through binding to fibrinogen that is also bound to the platelet αIIβ3. During adhesion to endothelium, platelets are activated and release proinflammatory cytokines, such as CD40L and interleukin-1β, that can further stimulate the endothelium. Adhesion of platelets or platelet-derived microparticles is followed by activation of endothelial nuclear factor-κB and nuclear factor-κB-regulated genes, such as monocyte chemoattractant protein-1, α,β1, intercellular adhesion molecule-1, and vascular cellular adhesion molecule-1, or genes that play important roles in chemotaxis and transmigration of monocytes.

Activated platelets also express and secrete the chemokines CCL5 (RANTES) and CXCL4 (platelet factor 4), which are deposited in a P-selectin–dependent manner on microvasculature, aortic endothelium, and monocytes. Deposition of these proinflammatory cytokines results in activation of monocyte integrins and in increased monocyte recruitment to atherosclerotic lesions.

Massberg et al showed in vivo that platelet adhere to carotid endothelium at an early stage of atherosclerosis in hypercholesterolemic apoE-deficient mice. Prolonged blockade of platelet adhesion by antibodies against GPIbα significantly attenuated atherosclerotic lesion formation in these animals, indicating that platelet GPIbα interaction with its ligands VWF and P-selectin, which are found on activated endothelial cells, might be responsible for platelet binding. This indicates that VWF could play an important role in thrombosis and hemostasis but could also be involved in atherosclerotic lesion development. Indeed, LDLR- or apoE-deficient mice that were also VWF-deficient showed a transient delay in atherosclerotic lesion development, with fewer monocytes in their lesions than wild-type control animals of the same background. Interestingly, absence of VWF affected mainly lesion distribution. VWF deficiency protected the animals from lesion development in areas with disturbed flow, such as arterial branch points of renal and mesenteric arteries and the aortic sinus. VWF may participate in the recruitment of monocytes directly through the VWF propolypeptide or indirectly by recruiting platelets that may facilitate leukocyte adhesion. There is also a possibility that VWF, through interaction with β3 integrin, is part of the mechanotransduction pathway mediating the shear stress responses in endothelium. Thus, the exact mechanism responsible for the bizarre lesion distribution in VWF-deficient mice remains to be established.

Platelets, once recruited to atherosclerotic lesions, contain a variety of molecules that can additionally promote chemotraction of leukocytes (platelet activating factor, macrophage inflammatory protein [MIP]-1α, and cationic proteins), stimulate smooth muscle cell and fibroblast proliferation (TGF-β3, platelet-derived growth factor, and serotonin), and promote collagen synthesis. Platelets with their cytokines thereby contribute directly to lesion progression and maturation.
already mentioned above, activated platelets also shed large amounts of soluble CD40L. 89 CD40L/CD40 interaction plays an important role in atherosclerosis because antibody to CD40L-inhibited lesion development in LDLR-deficient mice 90 and CD40L-deficient mice on apoE-deficient background was extensively protected from atherosclerosis. 91 With all of these activities of activated platelets, it is not surprising that repeated infusions of activated platelets promoted atherosclerosis in apoE/H/H mice. Here, too, the effect was dependent on the presence of platelet P-selectin. 84 Thus, 3 independent studies using very different experimental approaches and published almost simultaneously demonstrated that platelets and their adhesion receptors play an important role in atherosclerotic lesion formation. 72,84,85

Conclusions
Thrombosis and inflammation are intricately linked (Figure 3). The examples discussed above show that some of the same cellular and molecular players participate in both processes. On vascular injury, platelets cover the exposed subendothelial matrix and mediate additional platelet and leukocyte recruitment. To stop bleeding, they provide the required surface for the binding of leukocyte-derived microparticles containing tissue factor for a localized induction of the coagulation cascade. Platelets also release microparticles that mediate leukocyte-leukocyte and leukocyte–endothelial cell interactions. Most of these mechanisms play a role in inflammation as well. Activated platelets increase leukocyte adhesion to the endothelium and promote leukocyte activation through deposition of chemokines on the endothelium. This enables leukocytes to firmly attach to the vessel wall and finally to transmigrate into the subendothelial tissue (Figure 3). The role of platelets in chronic inflammatory diseases such as atherosclerosis has now been convincingly demonstrated. Thus, platelets are central to both thrombosis and inflammation.

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