Soluble Fibrin Is the Main Mediator of *Staphylococcus aureus* Adhesion to Platelets

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**Background**—Infective endocarditis (IE) caused by *Staphylococcus aureus* is associated with significant morbidity and mortality rates. Platelets play a dual role as adhesive cells forming associates with bacteria as well as specialized inflammatory cells. The specific role of the various factors involved in bacteria-platelet association has not yet been fully elucidated.

**Methods and Results**—We observed a dramatic increase in the capability to bind *S aureus* when platelets were activated with thrombin (from 5% to 30%, *P*<0.001). To pinpoint platelet-binding sites involved in the interaction, platelets from knockout mice and from patients with selective inherited deficiency of membrane proteins or of granules were used. CD36, GPIIb/IIIa, and P-selectin were excluded as receptors for *S aureus*. Platelets from patients with α-δ-storage pool disease and Gray platelet syndrome indicate the requirement of α-granule contents. Platelet activation by ADP did not promote platelet–*S aureus* associate formation, although these platelets were covered with bound fibrinogen. Only small numbers of associates between fibrinogen-covered bacteria and ADP-activated platelets were observed. Formation of fibrin alone was also not sufficient to induce association. Only when fibrin formation and platelet activation occurred together were large numbers of associates formed (*P*<0.001). A potential receptor for fibrin on *S aureus* is clumping factor A. Addition of thrombospondin-1 to control platelets increased the number of associates (*P*=0.02).

**Conclusions**—Soluble fibrin but not fibrinogen is the main mediator of platelet–*S aureus* association. In addition, platelet activation and the release of α-granule contents, particularly thrombospondin-1, is a requirement for platelet–*S aureus* association. *(Circulation. 2004;110:193-200.)*

**Key Words**: platelets ● endocardium ● fibrin ● infection

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*S aureus* is a highly virulent pathogen and a frequent cause of endovascular infections, such as intravascular catheter–related sepsis, and of infective endocarditis (IE). The mechanisms by which pathogens such as *S aureus* induce endovascular infection are complex and multifactorial. On the one hand, platelets have been thought to promote induction of endovascular infection and hematogenous dissemination of *S aureus* by providing the organism with an adherence surface on damaged endothelium. Diminished platelet binding in vitro by *S aureus* is associated with reduced virulence in a model of infective endocarditis.

On the other hand, platelets may also provide an important host defense function through release of platelet microbicidal proteins (PMPs). PMP-resistant strains can circumvent PMP activities at sites of vascular infections and have a survival advantage in terms of inducing IE. Patients with *S aureus* infective endocarditis complicating an infective intravascular device were significantly (~10-fold) more likely to have IE by a PMP-resistant strain.

For infections with PMP-resistant pathogens, the propathogenic adhesive role of platelets might outweigh their role as cells of specialized host defense.

New therapeutic strategies aiming at the inhibition of the adhesive interaction between PMP-resistant *S aureus* strains and platelets may be promising. This approach requires detailed understanding of the mechanism underlying this adhesion. To further characterize the pathogenic mechanisms, we examined the platelet-dependent factors that are involved in *S aureus* adhesion to platelets.

**Methods**

**Materials**
Bovine serum albumin, the ADP inhibitor apyrase, the fibrin formation–inducing enzyme ancrod, and α-thrombin were from Sigma. The thrombin inhibitor hirudin was from LOXO. The fibrin polymerizing inhibiting peptide GPRP (Gly-Pro-Arg-Pro) was from Calbiochem. Human fibrinogen was from Kordia. Monoclonal antibody against the platelet specific receptor CD42a (PE conju-
Platelets

All studies were performed with the blood donors giving informed consent and were approved by the local ethics committee. Blood donors had not taken any medication affecting platelet function for at least 2 weeks before the study. Platelet-rich plasma from trisodiumcitrate-anticoagulated blood was prepared by centrifugation. For one experiment, platelets were gel-filtered on Sephadex Cl-2B.8

Bacteria Preparation

Experiments were performed with either S aureus Newman, S aureus Cowan1, S aureus 4074 from a patient with endocarditis, or a clumping factor A (ClfA) deletion mutant of S aureus Newman (clfA/TwH17; DU58525) (2×10^8 cfu each) from fresh overnight cultures or for one experiment grown to exponential phase (OD_600 of 0.9) grown in brain-heart infusion media, washed in Tris-buffered saline (TBS pH 7.4), briefly sonicated (10 cycles of 1 second, 50 W, Branson), and diluted with TBS (containing 2 mmol/L Ca^2+) to 250 000 bacteria/μL. Microorganisms were labeled with the fluorescent dye Syto 13 (2 μmol/L, 10 minutes), washed in TBS/Ca^2+, and again briefly sonicated.

Fibrinogen Binding to S aureus

FITC-coupled fibrinogen was incubated with S aureus (120 000 cells/μL) in TBS for 15 minutes, and excessive fibrinogen was removed. After short sonification, fibrinogen-FITC binding was measured.

Measuring of Associate Formation of Platelets and S aureus

Platelets were diluted (25 000/μL) and labeled with an anti-CD42a PE-conjugated antibody. Labeled prepared cells were coincubated (10:1 S aureus:platelets) for 15 minutes at room temperature, and conjugate formation was measured immediately.

Labeled platelets in platelet-rich plasma were diluted, activated with α-thrombin or ADP for 3 minutes, and, subsequently, bacteria were added. To inhibit secondary thrombin formation, hirudin (10 U/mL) was added. To inhibit fibrin polymerization, experiments with either α-thrombin or with ancrod were performed in the presence of GPRP (1.25 mmol/L).8

To measure the effect of soluble fibrin, ancrord was added to platelets in fibrinogen-containing plasma for 3 minutes. The platelets were either left quiescent or activated with ADP for another 3 minutes, and microorganisms were added. These experiments were performed in the presence or absence of added Factor XIII (up to 3 U/mL).

Platelet-S aureus Associate Formation Using Platelets With Deficiencies in Membrane Glycoproteins or Granules

GPIIb/IIIa-deficient platelets were obtained from a patient with type I Glanzmann thrombasthenia (<1% GPIIb/IIIa), described previously.10

The CD36-deficient platelets of a blood donor (patient YA) and platelets from patient JE, who had the rare α-δ–storage pool disease, have been described previously.10,11 The platelets of patient JE did not contain any α-granules or any dense bodies.

Gray platelets from patients AP and WK have also been described previously. Both patients’ platelets did not contain any normal α-granules12 and were completely deficient in thrombospondin-1.

Blood from P-selectin knockout and wild-type mice was a kind gift from D. Vestweber, Muenster, Germany.

Platelet Aggregation

Staphylococcus aureus from overnight culture were washed and resuspended to an OD_{600} of 1.6 in HEPES-Tyrode buffer, pH 7.4. Bacterial suspension (50 μL) was added to gel-filtered platelets (450 μL, 2×10^9 platelets/mL). Platelet aggregation was assayed by light transmission at 37°C. Fibrinogen (100 μg/mL) and hirudin (10 U/mL) were incubated with the platelets before addition of S aureus and/or ancrord (0.05 U/mL).

Cell Sorting

Bacteria-platelet suspension, prepared as described above, was fixed with 0.5% formaldehyde. Bacteria-positive platelets were measured and sorted with the use of a MoFlo High-Performance Cell Sorter (Dako-Syotoman). Different sorts were examined by light and fluorescence microscopy.

Transmission Electron Microscopy

Platelets were rapidly frozen without prior fixation, freeze-substituted, and embedded in araldite as described previously.12 Serial ultrathin sections were stained with uranyl acetate and lead citrate.

Platelet-S aureus associates were fixed with Karnowsky’s solution (4% parafomaldehyde, 5% glutaraldehyde in 0.2 mol/L cacodylate buffer). Small blocks of the centrifugation pellets were washed in buffer, postfixed with osmium tetroxide after Caulfield, and embedded in araldite. Serial ultrathin sections were stained.

Statistical Analysis

The results are expressed as mean±SD. Statistical significance was determined by using the unpaired Student’s t test and ANOVA. Significance level was defined as P<0.05.

Results

Platelet Activation With Thrombin But Not With ADP Increased the Rate of Platelet–S aureus Associates

Platelets bound to S aureus in a specific manner (Figure 1A). Cell sorting of bacteria-positive platelets showed that 1 bacterium per platelet is enough for it to be considered bacterium-positive. Most platelets carried more than 1 bacterium (Figure 1B). Transmission electron microscopy showed that the bacteria are found attached to strongly activated and degranulated platelets (Figure 1C) Thrombin activation of platelets significantly (P<0.001) increased the rate of platelet–S aureus associate formation in a dose-dependent manner (Figure 1D). In contrast, ADP activation in the presence of hirudin, to prevent secondary thrombin activation, had almost no effect.

Platelet–S aureus Associate Formation Does Not Depend on CD36 or GPIIb/IIIa

The number of platelet–S aureus associates did not differ between CD36-deficient and control platelets (Figure 2A).

Platelet GPIIb-IIIa deficiency did not yield significantly different numbers of conjugates with quiescent or thrombin-activated platelets (Figure 2A, P=0.11).

Granule Proteins Are Needed for Platelet–S aureus Associate Formation

Platelets completely deficient in both α-granules and dense bodies generated very low numbers of associates (<3%) with S aureus. In sharp contrast to controls, the number of associates did not markedly increase when platelets were activated with thrombin (Figure 2A).

To confirm the role of the storage granules and to discriminate whether the α-granules or the dense bodies are more important, we studied platelets from patients with Gray
platelet syndrome. Only remnants of α-granule membranes existed in these platelets (Figure 2B), and they were completely deficient in thrombospondin-1. Again, the basic number of platelet–S aureus associates formed was very low and could not be increased by activation of the platelets with thrombin (Figure 2C).

The α-Granule Membrane Protein P-Selectin (CD62) Is Not Involved in Platelet–S aureus Associate Formation

P-selectin (CD62) is an α-granule membrane protein that mediates platelet-leukocyte associate formation. In comparison to wild-type (SV 129) mice platelets, platelets from
P-selectin knockout mice did not differ in their capability to bind *S. aureus*. The association rate of thrombin-activated (1 U/mL) platelets was 47.67 ± 15.32% (wt) versus 47.78 ± 13.14% (CD62<sup>−/−</sup>).

**Figure 2.** Platelet–*S. aureus* associate formation needs α-granule proteins and does not depend on CD36 or GPIIb-IIIa. A, Number of platelet–*S. aureus* Cowan1 associates did not differ between CD36-deficient, GPIIb/IIIa-deficient, and control platelets, neither with nor without thrombin activation. However, platelets from patient JE, deficient in α-granules and dense bodies, built very low numbers of associates with *S. aureus* Newman, and the number increased only slightly after thrombin activation. B, Transmission electron microscopy of section of a normal platelet (MT, marginal microtubules; G, α-granules; M, mitochondrion; SCS, surface-connected system; DTS, dense tubular system; DB, dense bodies). In transmission electron microscopy of a section of a Gray platelet, no characteristic profiles of α-granules are visible; several α-granules show electron lucent matrix or are small in dimension (iG). C, Platelets from patient AP and patient WK with Gray platelet syndrome, deficient in thrombospondin-1, formed low numbers of platelet–*S. aureus* associates, and the number was not increased by activation of platelets with thrombin. Quiescent platelets (gray columns); thrombin-activated (0.2 U/mL) platelets (black columns). PRP indicates platelet-rich plasma; n = 3.

**Soluble Fibrin But Not Fibrinogen Is the Main Mediator of Platelet–*S. aureus* Associate Formation**

The main difference in platelet activation with thrombin and ADP is that thrombin, in addition to being a platelet agonist,
cleaves fibrinogen to form fibrin. We therefore analyzed whether fibrin formation is sufficient to induce platelet–Staphylococcus aureus association formation using ancrod, an enzyme that cleaves fibrinopeptides from the α-chain of fibrinogen without inducing platelet activation. Formation of soluble fibrin by ancrod in the presence of quiescent platelets did not induce association formation (Figure 3A). Activation of platelets with ADP in the presence of fibrinogen was sufficient to induce full binding of fibrinogen (Figure 3B), and all S aureus strains were able to bind fibrinogen (Figure 3C), but this did not result in platelet–S aureus association formation. In addition, when fibrinogen-covered bacteria were added to ADP-activated platelets, only a minor increase in number of bacteria-positive platelets was observed (Figure 3D). Only when platelets were activated with ADP and fibrin was formed were large numbers of associates formed in a fibrin concentration–dependent manner (Figure 3E, \(P<0.001\)). Addition of FXIII did not show an effect on associate formation (data not shown). The same phenomenon was observed with the use of the clinically derived S aureus strain (4074) from a patient with endocarditis and S aureus strain Newman in log phase instead of stationary phase (Figure 4).

Figure 3. Soluble fibrin but not fibrinogen is the main mediator of platelet–S aureus associate formation. A, Soluble fibrin was formed, without activation of the platelets, with the use of ancrod. Ancrod was added to platelets in fibrinogen-containing plasma, and S aureus Cowan1 was added thereafter. No associate formation was recognized in the presence of soluble fibrin. GPRP prevented fibrin polymerization. B, ADP induced fibrinogen binding to platelets. With 2 \(\mu\)mol/L ADP, full binding response was observed. As shown in Figure 1D, these ADP-activated, fibrinogen-carrying platelets bound only a small amount of S aureus. C, S aureus Newman (solid line), S aureus Cowan1 (dashed line), and S aureus 4074 (dotted line) were able to bind fibrinogen (up to 20 000 molecules). D, Nonactivated platelets did not bind S aureus Cowan1, which were loaded with 300 \(\mu\)g/mL fibrinogen. Also, ADP-activated (2 \(\mu\)mol/L) platelets built very low numbers of associates with fibrinogen-covered S aureus Cowan1. ADP-activated (2 \(\mu\)mol/L) platelets bound high amounts of S aureus Cowan1 in presence of fibrin, formed by 0.4 U/mL ancrod. E, Soluble fibrin mediates associate formation between ADP-activated (2 \(\mu\)mol/L) platelets and S aureus Cowan1 in a dose-dependent manner (200 \(\mu\)g/mL fibrinogen; 10 U/mL hirudin, 1.25 mmol/L GPRP). PRP indicates platelet-rich plasma; n=3.
Clumping Factor A Is Involved in Platelet–S aureus Associate Formation

To determine whether the adhesin ClfA is involved in fibrin-mediated platelet–S aureus associate formation, we used a ClfA-deficient mutant of S aureus Newman. Significantly fewer platelet–S aureus associates were formed after activation of platelets and fibrin formation in comparison to controls (black columns). PRP indicates platelet-rich plasma; n=3.

Formation of Soluble Fibrin Led to Platelet Aggregation Induced by S aureus

Association of bacteria with platelets led to the next step in pathogenesis, platelet aggregation. As shown in Figure 6, formation of soluble fibrin is the key mechanism allowing big aggregates to be built.

TSP-1 Addition Increased Number of Platelet–S aureus Associates

The most abundant protein from platelet α-granules is the adhesive protein thrombospondin-1 that binds after platelet activation back to the surface. Because the patients with Gray platelets, deficient in thrombospondin-1, were not available an additional time, we added exogenous thrombospondin-1 to the plasma of healthy donors. A significantly increased number of associates (Figure 7, thrombin activation: P=0.038, ADP and ancrod: P=0.02) was observed.

Discussion

We have demonstrated that in contrast to current opinion, soluble fibrin but not fibrinogen is the main mediator of S aureus adhesion to platelets in solution and that α-granule proteins play a major role in associate formation.

We observed a dramatic increase in the capability to bind S aureus when platelets were activated. This finding clearly shows that activation-dependent processes play a major role in platelet–S aureus adhesion. To pinpoint the binding sites of the platelets involved in the interaction, platelets from knock-out mice or from patients with rare selective inherited deficiencies of membrane proteins or of granules were used.

From our studies, both CD36 and P-selectin can be excluded as receptors for S aureus. CD36-deficient human platelets bound normal numbers of S aureus, and platelets from P-selectin knockout mice behaved like their wild-type controls. Surprisingly, the platelet receptor for fibrinogen (GPIIb/IIIa) appeared to have no role in platelet–S aureus adhesion in solution. This finding is in accordance with the observations of Bayer et al., demonstrating that S aureus induces platelet aggregation through a fibrinogen-dependent mechanism that is independent of glycoprotein IIb/IIIa fibrinogen–binding domains. On first look, our findings appear to be in contrast to those found by O’Brien et al., who identified the bacteria proteins involved in associate formation in an elegant way. The main difference between our work and that of O’Brien is that we used hirudin to inhibit the action of thrombin. In the experimental setting of O’Brien
aggregate formation by *S. aureus* takes several minutes. This observation can easily be explained, assuming that *S. aureus* activates platelets, and this activation process then leads to thrombin formation as described by Arvand et al and in this way to aggregation caused by thrombin. Thrombin-induced platelet aggregation is known to be inhibited by GPIIb/IIIa antagonists. Therefore, GPIIb/IIIa might be important for secondary aggregation by *S. aureus*. Platelet and *S. aureus* instantly stick together, and *S. aureus* immediately leads to platelet aggregation if fibrin is already present (upper curve in Figure 6).

The secretion process must be of high relevance for associate formation in solution. Platelets, deficient in both α-granules and dense bodies, promote only very low numbers of associates with *S. aureus*, and the numbers could not markedly be increased by thrombin activation. To answer the question of which of the two storage granules is the most important, we studied Gray platelets. Patients’ platelets showed very little associate formation, and this process was not increased by thrombin activation. These findings clearly demonstrate that α-granule proteins are necessary to induce platelet–*S. aureus* associate formation.

When given with antimicrobial therapy, aspirin not only reduced vegetation weight but also improved the rate of sterilization in a rabbit model of *S. aureus* endocarditis. The same authors also showed that a combination of aspirin and ticlopidine act synergistically to optimally reduce the weight of aortic valve vegetation in the same animal model of endocarditis. The effects were significant but still weak (*P* = 0.043). Both drugs, aspirin and ticlopidine, have some but not profound inhibitory effect on platelet secretion by strong agonists such as thrombin.

In contrast to thrombin activation, platelet activation by ADP did not promote platelet–*S. aureus* associate formation. The main difference between thrombin and other platelet agonists is the property of thrombin to cleave fibrinogen, resulting in generation of fibrin. Formation of soluble fibrin alone was not sufficient to induce *S. aureus* adhesion to quiescent platelets, and activation of platelets by ADP alone was also not sufficient to induce *S. aureus* adhesion to platelets, although these platelets were covered with bound fibrinogen. In addition, fibrinogen-covered bacteria did not bind to quiescent platelets, and with ADP-activated platelets, only a small increase in the number of associates was observed. Only when fibrin formation and platelet activation occurred together, a huge number of associates between *S. aureus* and platelets were formed. Factor XIII did not increase the number of associates, demonstrating that stable cross-linking is not necessary for this process.

One of the main differences between fibrinogen and soluble fibrin is that fibrin in solution offers repetitive binding sequences for ligands. Repetitive binding sequences increase the avidity of binding between proteins and in this way strengthen the binding forces, as would a Velcro fastener. The binding of *S. aureus* to adhered platelets and to adhered proteins is different from the binding in solution because immobilization mimics repetitive sequences. If fibrinogen or von Willebrand factor are immobilized, *S. aureus* can bind to it (as shown previously).

Experiments with a ClfA-negative construct of Newman suggest that this adhesin, in addition to its known property to bind fibrinogen, mediates in part the binding of *S. aureus* to platelets by fibrin. As residual interaction is observed, however, other adhesins such as ClfB or FnBPA might contribute to fibrin binding.

Thrombospondin-1 is released from α-granules and binds back to platelet surfaces and to immobilized fibrinogen or to un–cross-linked soluble fibrin. Addition of thrombospondin-1 to activated platelets led to a significant increase in the number of associates. Thrombospondin-1 is known to bind to activated platelets as well as to *S. aureus*. In addition, fibrin binds to activated platelets and to *S. aureus*. Together, both proteins can form large binding clusters to promote the adhesion between the two cells. A hypothetical model is given in Figure 8.

Preventing this interaction might be a new avenue to treat endovascular infections. On the basis of these new results, the inhibition of thrombin function might be a more efficient strategy to inhibit platelet–*S. aureus* association than treatment with aspirin and/or ticlopidine. Aspirin as well as ticlopidine are only weak inhibitors of platelet secretion processes induced by strong agonists. On the basis of our results, direct thrombin inhibitors, administered together with antibiotics, might be more effective compounds to prevent and treat *S. aureus* endovascular infections because they directly inhibit both fibrin formation as well as activation of platelets and secretion of thrombospondin-1.

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