Platelet activation leads to activation and propagation of the complement system

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Inflammation and thrombosis are two responses that are linked through a number of mechanisms, one of them being the complement system. Various proteins of the complement system interact specifically with platelets, which, in turn, activates them and promotes thrombosis. In this paper, we show that the converse is also true: activated platelets can activate the complement system. As assessed by flow cytometry and immunoblotting, C3 deposition increased on the platelet surface upon cell activation with different agonists. Activation of the complement system proceeded to its final stages, which was marked by the increased generation of the anaphylotoxin C3a and the C5b–9 complex. We identified P–selectin as a C3b–binding protein, and confirmed by surface plasmon resonance binding that these two proteins interact specifically with a dissociation constant of 1 μM. Using heterologous cells expressing P–selectin, we found that P–selectin alone is sufficient to activate the complement system, marked by increases in C3b deposition, C3a generation, and C5b–9 formation. In summary, we have found that platelets are capable of activating the complement system, and have identified P–selectin as a receptor for C3b capable of initiating complement activation. These findings point out an additional mechanism by which inflammation may localize to sites of vascular injury and thrombosis.

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Abbreviations used: CR, complement receptor; CHO, Chinese hamster ovary; CHO-P; CHO expressing P-selectin; HUS, hemolytic uremic syndrome; PNH, paroxysmal nocturnal hemoglobinuria; PPP, platelet-poor plasma; PRP, platelet-rich plasma; TRAP, thrombin receptor–activating peptide.

Complement is a multi-component system that involves several plasma and cell-expressed proteins, and has been conserved throughout evolution. Although recognized primarily as an important part of innate immunity, complement has adopted additional functions in different organisms. The complement system mediates cell–cell interactions in many physiological processes, such as hematopoiesis, organogenesis, and in reproduction (1). For these functions to be executed, the complement system requires activation that is marked by the formation of the C3 convertase complex on the surface of target cells. This convertase cleaves large quantities of C3 into C3b, the main effector of complement, and C3a, a potent mediator of inflammation released into the medium. C3b and its degradation products fixed on target cells functions as opsonins, thereby marking the cell for destruction by phagocytes attracted by anaphylotoxins (C3a and C5a). Also, C3b on the cell surface participates in the serial activation of complement proteins (C5–C9) that polymerize and form the C5b-9 complex, also known as the membrane attack complex. If an adequate number of C5b-9 complexes form, the target cell is destroyed.

Several negative regulatory proteins exist to prevent uncontrolled activation of the complement system and widespread tissue damage. A deficiency in the negative regulators of complement leads to excessive complement activation, as observed in paroxysmal nocturnal hemoglobinuria (PNH) and atypical or familial hemolytic uremic syndrome (HUS). Interestingly, both of these diseases are characterized by thrombosis. Studies suggest that the mechanisms of thrombosis in PNH and familial HUS are related to platelet activation induced by complement activation. Patients with PNH were found to have baseline platelet hyperreactivity, as evidenced by increased P–selectin expression (2). Also, mice deficient in the negative regulator of complement, CD59b, exhibit platelet hyperreactivity (3). Much experimental evidence supports the notion that complement activation leads to platelet activation. Several proteins of the complement system, including C3 and C5–C9, were shown to potentiate thrombin-induced platelet secretion and aggregation (4). The anaphylotoxin C3a was found to induce platelet activation and aggregation (5). Also, treatment of platelets with

sublytic concentrations of C5b-9 caused transient membrane depolarization (6), granule secretion (7), generation of procoagulant platelet microparticles (8), and translocation of phosphatidylserine to the outer membrane leaflet (9, 10) Collectively, these studies make the case that the complement system may serve as a link between inflammation and thrombosis, and reveal specific interactions between complement proteins and platelets.

We determined whether a reciprocal interaction also exists, whereby platelets can activate complement. Here, we show that upon activation, platelets activate the complement system, which proceeds to its final stages marked by the formation of the C5b-9 complex. We have also identified P-selectin expressed on activated platelets as a receptor for C3b, thus linking thrombosis and local activation of the complement system on platelets.

RESULTS

C3 deposition on activated platelets

We determined whether complement activation marked by C3 deposition increased on shear-activated platelets. We subjected platelets in plasma to shear rates of either 0, 1,000, or 10,000 s^{-1} for 1 min; rates that would be expected in blood stasis, arterioles, and in stenotic vessels, respectively. The amount of C3 deposition on platelets increased with increasing shear rates (Fig. 1 A). Under static conditions, platelets contained appreciable quantities of C3, an amount that increased when platelets were subjected to a shear rate of

1000 s^{-1}, and which was greatest at a shear of 10,000 s^{-1}. These differences were specific, as the amount of the platelet protein, β-actin, was similar in the three samples.

We determined whether C3 is also deposited on platelets activated with other agonists. Compared with unstimulated platelets, platelets activated with either ADP or thrombin receptor–activating peptide (TRAP) had a larger amount of associated C3 (Fig. 1 B). Similar amounts of the platelet receptor GP Ibα in the three samples indicated equal protein loading.

C3a generation in activated platelets

As an additional test of whether platelets activate complement, we examined generation of C3a, which is the released cleavage product of C3. Platelets (250 × 10^3/μl) subjected to shear rates of 1000, 5000, and 10,000 s^{-1} for 60 s generated on average 15, 46, and 245 ng C3a/ml platelet-rich plasma (PRP), respectively, than platelets in static conditions (Fig. 2 A). We also tested the effect of agonists on C3a generation by platelets. Platelets that were activated with either collagen or TRAP in the presence of 1 ml of plasma generated on average 169 and 89 ng C3a, respectively, which was
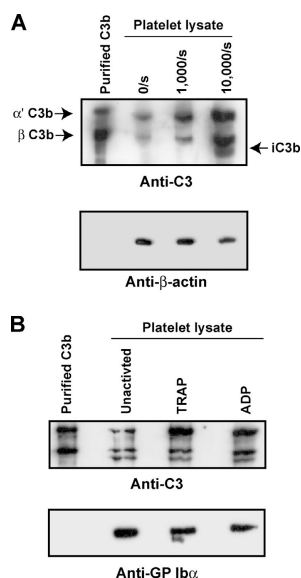

Figure 1. Deposition of C3 on activated platelets. Platelet-rich plasma was (A) subjected to different shear rates for 60 s, or (B) activated with different agonists. Platelets were then pelleted, washed with TBS buffer, lysed in reducing SDS sample buffer, and subjected to PAGE and Western blotting for C3. The same membranes was stripped and probed for β-actin or GP Ibα as loading controls. These results are representative of three experiments.
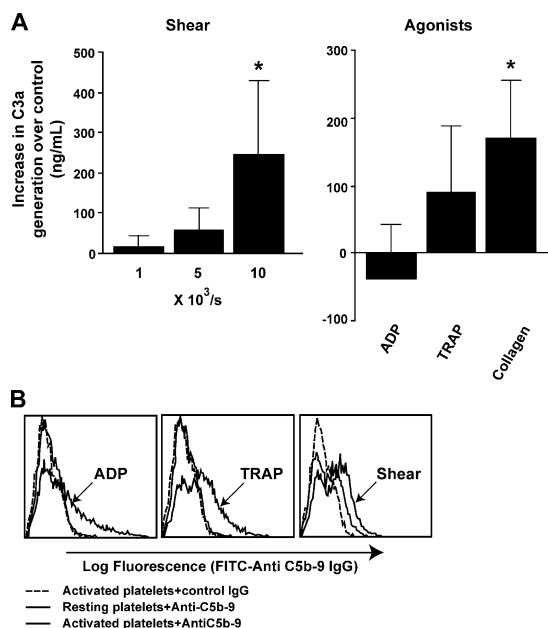

Figure 2. Activation of complement on activated platelets. (A) Generation of C3a by activated platelets. 1 ml PRP was stimulated with either 20 μM ADP, 10 μM TRAP, 20 μg/ml collagen, or with different shear rates for 60 s. Platelets were pelleted, and C3a levels were measured in the plasma supernatant using ELISA. The plasma of a suspension of resting platelets served as the controls. $n = 4$; *, $P < 0.05$. (B) Formation of the C5b-9 complex on activated platelets: platelet-rich plasma (PRP) was stimulated with either 20 μM ADP, 10 μM TRAP, or 10,000 s^{-1} × 60-s shear. Platelets were then probed by flow cytometry for the presence of the C5b-9 complex using a rabbit anti–C5b-9 neoepitope. Baseline fluorescence was set using nonimmune rabbit IgG and a secondary FITC-conjugated antibody. These results are representative of four experiments.

more than unstimulated platelets (Fig. 2 a). In contrast, stimulation of platelets with ADP did not significantly affect C3a generation. We excluded the possibility that contaminating neutrophils contributed to C3a generation by adding increasing counts of autologous neutrophils (from 500 to 5×10^5 neutrophils) to a constant number (2×10^5) of platelets in plasma, which were subsequently stimulated with collagen. We found no differences in the amount of C3a generation throughout the entire range of neutrophil counts tested (unpublished data).

C5b–9 complex forms on activated platelets

We investigated whether complement activation on the platelet surface proceeded to its final stages, which is marked by the formation of the membrane attack complex (C5b-9 complex). Compared with resting platelets, platelets activated with either 20 µM ADP, 5 µM TRAP, or shear exhibited greater amounts of C5b-9 on their surface, relative to unstimulated platelets (Fig. 2 B).

C3b binds to activated platelets

To study the molecular events involved in the initiation and propagation of the complement activation on the surface of platelets, we measured the deposition of FITC-conjugated C3b on resting and TRAP-activated washed platelets. On average, <10% of unstimulated platelets bound C3b-FITC. However, there was a marked increase in the binding of C3b-FITC when platelets were stimulated with TRAP, with >65% of platelets becoming positive for C3b-FITC (Fig. 3 A). As an additional control, we determined the binding of C3b-FITC to the monocytic cells, THP-1, which are known to constitutively express CR1 (11), a receptor for C3b, and CR2 (12). On average, 77% of THP-1 cells bound C3b-FITC (Fig. 3 B).

To determine whether C3b was binding a platelet protein, we removed surface proteins from activated platelets using a low concentration of protease K (50 µg/ml). After extensive washing, platelets were probed with FITC-labeled C3b. Removal of surface proteins abolished binding of C3b to the surface of activated platelets (Fig. 3 C). As assessed by global protein tyrosine phosphorylation profiles (13), TRAP-activated platelets that were subsequently treated with protease K maintained their activated state (unpublished data).

P–Selectin mediates C3b binding to activated platelets

Platelets express P-selectin upon activation, and P-selectin contains nine sushi domains, which are a common structural motif present in many complement-binding proteins, such as complement receptors (CR) 1 and 2. We therefore examined whether P-selectin is the C3b receptor on activated platelets. We investigated the effect that an anti–P-selectin antibody had on C3b binding to activated platelets. Treatment of TRAP-activated platelets with an anti–P-selectin antibody significantly reduced the amount of deposited C3b (Fig. 3 D).
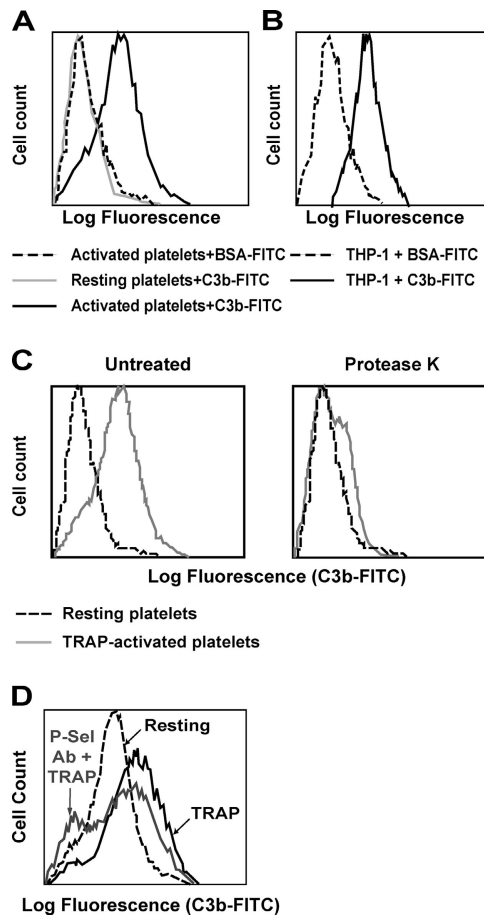

Figure 3. Binding of C3b to activated platelets. (A) Binding of C3b to the surface of activated platelets was measured by flow cytometry using FITC-conjugated purified C3b. Washed platelets, either resting or stimulated with 5 µM TRAP, were probed with FITC-C3b. Background fluorescence was set using BSA-FITC. (B) Binding of C3b-FITC to the monocytic cells, THP-1, which constitutively express the complement receptor CR1. (C) Cleavage of surface proteins inactivated using 50 µg/ml protease K abrogates the binding of FITC-conjugated C3b. (D) Binding of FITC-C3b to TRAP-activated platelets was inhibited by treating the platelets with anti–P-selectin antibody.

P–Selectin mediates the adhesion of activated platelets to C3b under flow conditions

To test the interaction between P-selectin and C3b under flow conditions, we used a flow chamber apparatus and perfused TRAP-activated platelets over immobilized C3b at a constant shear stress of 2 dynes/cm². Activated platelets readily formed aggregates over the C3b as early as 20 s of perfusion time, developing into large platelet thrombi within 2 min. This interaction was specific, because activated platelets did not attach on an albumin–coated surface (unpublished data). Pre-treatment of the platelets with an anti–P-selectin antibody almost completely eliminated thrombus formation, confirming that the interaction between activated platelets and C3b is mediated by P-selectin (Fig. 4). In contrast, pretreatment of platelets with an antibody against the
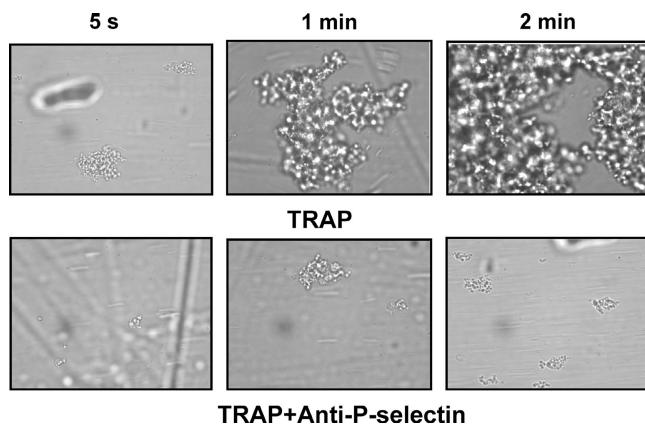
Figure 4. P–Selectin mediates the interaction between activated platelets and C3b. TRAP-activated platelets were perfused over C3b immobilized on a glass coverslip (30 μg/ml) at a constant shear stress of 2 dyne/cm² in a parallel plate flow chamber. Thrombus formation was visualized and recorded over 2 min. Treatment of platelets with an anti–P-selectin polyclonal antibody (25 μg/ml) abolished platelet adhesion to the immobilized C3b. Activated platelets failed to adhere to immobilized BSA. All the images are taken with 20× magnification.

platelet receptor GPIbα did not inhibit the binding of activated platelets to C3b (unpublished data).

C3b binds to Chinese hamster ovary (CHO) cells expressing P-selectin (CHO–P)

We measured the binding of plasma C3 to CHO and CHO-P cells. We incubated the cells in fresh human plasma and measured the amount of deposited C3 using a polyclonal anti-C3 antibody and flow cytometry. Compared with CHO cells, CHO cells expressing P-selectin bound more plasma C3 (Fig. 5 A). To further examine P-selectin as a C3b-binding protein in an isolated system, we measured the binding of FITC-conjugated C3b to CHO-P cells. Compared with CHO cells, CHO-P cells bound much more C3b: 87% of CHO-P cells were positive for C3b-FITC compared with 2% of CHO cells. These differences in C3b binding also resulted in a 7.7-fold greater C3b-FITC mean fluorescence intensity (MFI) in CHO-P compared with control CHO cells (MFI, 476 vs. 62 in CHO-P and CHO cells, respectively; $n = 5$, $P < 0.01$; Fig. 5 B).

C3b binding to P-selectin was partially dependent on calcium and magnesium. In the absence of these cations, binding of C3b to P-selectin was ∼5.7-fold greater than to cells expressing a control protein (the βIX subunits of the GPIb–IX-V complex). However, in the presence of 2 mM CaCl₂, 1 mM MgCl₂, or 2 mM CaCl₂ + 1 mM MgCl₂, the binding of C3b to P-selectin increased by ∼33%, 55%, and 63%, respectively, compared with the sample without calcium or magnesium (Fig. 5 C). For these experiments, we used two negative controls: first, CHO-P cells incubated with FITC-conjugated BSA; and second, binding of C3b-FITC to CHO cells expressing the βIX subunits of the
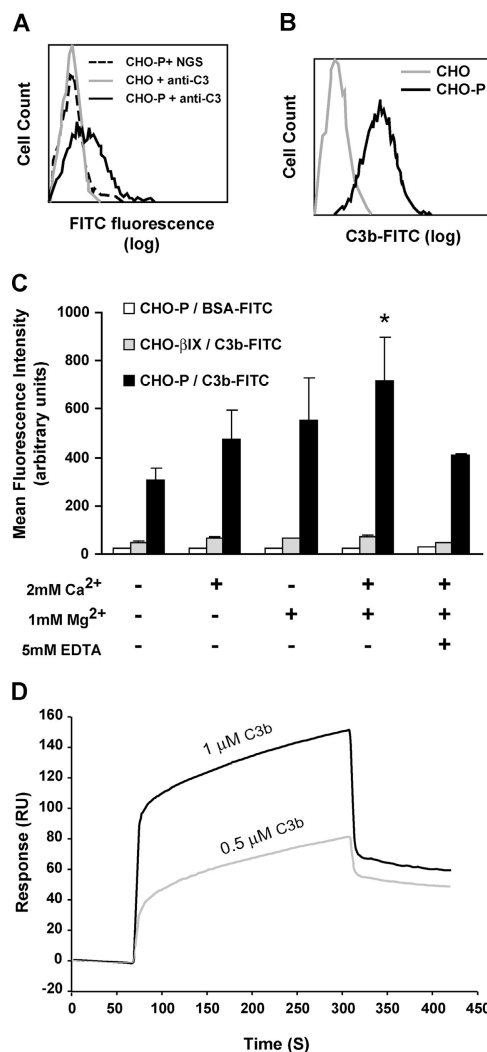

Figure 5. Binding of C3b to P-selectin. (A) Binding of plasma C3 to CHO-P and CHO cells was studied by incubating the cells in platelet-poor plasma for 30 min at 37°C. Cells were then probed with goat anti-C3 antiserum and a secondary FITC-anti–goat antibody. Baseline fluorescence was set using normal goat serum (NGS) and the secondary antibody. (B) Binding of FITC-labeled purified C3b was determined in CHO and CHO-P by flow cytometry. (C) The effect of calcium and magnesium on the binding of C3b to P-selectin expressed on CHO cells (CHO-P) was assessed by flow cytometry. Binding of FITC-labeled bovine serum albumin (BSA-FITC) to P-selectin, and binding of C3b-FITC to cells expressing the βIX subunit of the GPIb–IX-V complex served as negative controls. $n = 4$; *, $P < 0.05$. (D) Surface plasmon resonance binding studies of the interaction between purified C3b and recombinant ectodomain of P-selectin. Binding curves of P-selectin were obtained perfusing different concentrations of purified C3b over the P-selectin surface, as described in the Materials and methods. Dissociation constant (K_d) obtained for binding of C3b to P-selectin was 1.0 μM. RU, resonance units.

platelet GPIb–IX-V complex. Because GPβIX is expressed on the platelet surface, but has no known ligand-binding properties (14), it served as a negative control for C3b binding. By flow cytometry, CHO-βIX cells did not bind C3b-

FITC above a control set with equal concentrations of the control protein, BSA-FITC (unpublished data).

Surface plasmon resonance analysis of the C3b–P-selectin interaction

To assess the specificity of the P-selectin–C3b interaction, we used surface plasmon resonance analysis in which the P-selectin was immobilized on a CM5 chip. Various concentrations of C3b ranging from 0.5 to 5.0 μM were perfused over the immobilized P-selectin. Analysis of the sensograms yielded a K_d of 1 μM (Fig. 5 D).

P-Selectin activates the complement system

C3a generation. The data described before identified P-selectin as a C3b-binding protein. Therefore, we determined whether P-selectin is also capable of activating the complement system. We tested C3a generation in CHO and CHO-P cells by incubating them for 1 h in normal, cell-free plasma. Cells expressing P-selectin generated a significant 77% more C3a than did mock-transfected cells (1,240 vs. 697 ng/ml; Fig. 6 A).

C5b-9 formation. We next determined whether the formation of the terminal complement complex (the C5b-9 complex), was greater on CHO-P cells than on CHO-cells. Cells were incubated in cell-free plasma for 1 h, at 37°C, and C5b-9 formation was subsequently measured by flow cytometry. Compared with control CHO-cells, cells expressing P-selectin demonstrated an increase in the number of C5b-9 complexes formed on their surfaces (Fig. 6 B).

C3 convertase and P-selectin. To determine whether factor B is necessary for P-selectin–expressing cells to activate complement, we reconstituted the common pathway of the complement system, with or without factor B, and measured the ability of CHO-P cells to generate C3a as a surrogate of complement activation. CHO-P cells, but not control CHO cells, generated significantly greater amounts of C3a in the presence of factor B than in its absence (1,780 vs. 1,093 ng/ml, $n = 4$, P = 0.01) (Fig. 6 C).

DISCUSSION

Thrombosis and inflammation coexist in many physiologic and pathologic processes. Several mechanisms link these two processes, such as the binding of leukocytes to activated platelets at sites of thrombosis (15), the release of proinflammatory chemokines and cytokines by activated platelets (16–18), and the cross-linking of CD40 and CD40L between platelets and leukocytes (19). Working together, these two systems ensure that the tissue repair responses localize to sites of injury, where hemostasis has taken place.

Activation of the complement system during inflammation is likely to be a significant contributor to the prothrombotic drive. The direct products of complement activation, the membrane attack complex C5b-9, and the anaphylotoxins C3a and C5a, have been shown to activate platelets (5)
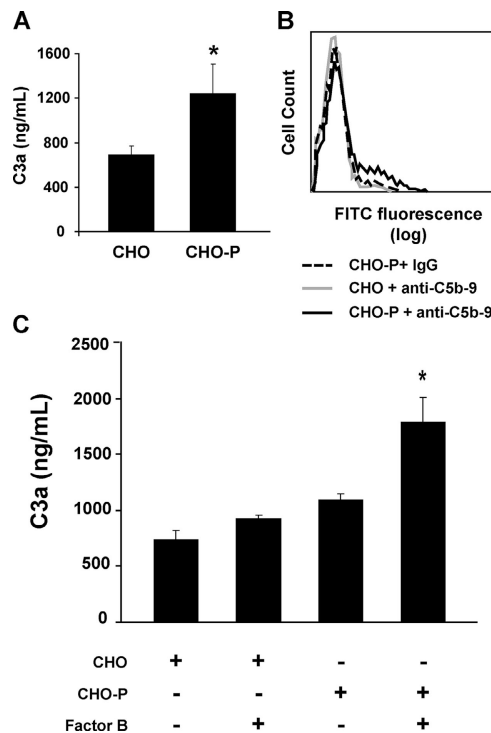
Figure 6. P-Selectin activates the complement system. P-Selectin–mediated activation of the complement system was studied in CHO cells. (A) CHO-P and CHO cells were incubated in PPP for 60 min. The cells were then sedimented, and C3a was measured in the plasma supernatant by ELISA. $n = 4$; *, P < 0.05. (B) Formation of the C5b-9 complex on CHO-P and CHO cells was measured by flow cytometry using a rabbit anti–C5b-9 neoepitope. Baseline fluorescence was set using nonimmune rabbit IgG. These results are representative of four other experiments. (C) CHO and CHO-P cells were incubated in a reconstituted complement system, with or without factor B, for 60 min at 37°C. Cells were then sedimented and C3a was measured by ELISA in the supernatants. $n = 4$; *, P < 0.05.

and promote coagulation by stimulating phosphatidylserine exposure (9, 10). Thus, it is not surprising that dysregulated complement activation, as observed in PNH and HUS, is characterized clinically by thrombotic events. Conversely, a deficiency in complement proteins may cause bleeding.

A setting in which complement-mediated platelet activation appears to play a major role is in immune complex-induced glomerulonephritis. Localization of platelets in renal glomeruli was shown to enhance inflammation and renal injury in an animal model of glomerulonephritis (20). This process seemed to depend on both complement proteins and platelets, as depletion of either of them significantly reduced kidney damage (20, 21).

Here, we show an additional link between thrombosis and inflammation. We show that activated platelets are capable of activating the complement system through a process that is partially dependent on P-selectin. Activated platelets acquired significant amounts of C3 in the plasma, which is an effect that occurred with a variety of agonists, including

TRAP, ADP, and shear. Lysates of unstimulated platelets were found to contain significant quantities of C3. It is likely that most of this C3 was stored in α-granules, and that the increase in C3 upon platelet activation represented deposition of the protein on the platelet surface. Interestingly, an increase in C3 deposition was observed with increasing shear rates, suggesting that the amount of deposited C3 is activation dependent. We confirmed that C3b binds to the surface of activated platelets. Consistent with the activation of complement on platelets, we found that platelets activated with either shear or collagen generated >200 ng/ml (∼24 nM) C3a above the baseline concentrations. Considering that C3a is a potent anaphylotoxin capable of inducing inflammation at concentrations in the nanomolar range (22), a 200-ng/ml increase in C3a concentrations is likely to be biologically important. The finding that activated platelets generate C3a is consistent with the study of Weksler and colleagues, who showed that activated platelets produced a chemotactic stimuli for neutrophils in an in vitro model of transendothelial migration (23).

We found that complement activation induced by activated platelets proceeded to its terminal phases, as evidenced by the formation of the C5b-9 complex. Because C5b-9 itself can potently activate platelets, the finding that activated platelets lead to C5b-9 formation points out a potentiating mechanism for platelet activation and thrombosis that is mediated by the complement system.

Our studies shed light on the results reported by Polley and Nachman in 1978, in which they found increased deposition of C3 and C5b-9 on platelets stimulated with thrombin (4). Because deposition of these complement proteins occurred even in the absence of factor B or C2, the authors postulated that there was a distinct mechanism of complement activation that was independent of the alternative and classic pathways, which require factor B and C2, respectively. In the model they postulated, a platelet protein, in conjunction with thrombin, mediated the cleavage of C3 resulting in complement activation. Currently, it is known that platelets store significant amounts of complement proteins within their granules, and this explains why the absence of factor B or C2 in the platelet suspension would not prevent complement activation. Our results indicate that complement activation by stimulated platelets is not restricted to a particular agonist. However, whether platelets stimulated by different platelet agonists differ in their ability to activate the complement system is not known and cannot be determined from our studies. Despite several experiments and using platelets from different donors, we found no significant differences in C3a generation between resting and ADP-activated platelets. We have no explanation for these findings but we believe that, similar to collagen- and TRAP-activated platelets, ADP-stimulated platelets are capable of activating the complement system on their surfaces. This is evidenced by increased deposition of C3b and increased formation of the C5b-9 complex on ADP-stimulated platelets (Figs. 1 B and 2 B).

We investigated the mechanisms by which the surfaces of activated platelets become permissive for complement activation. Our initial results indicated that the surface molecule that bound C3b was activated or expressed only upon platelet activation, and, obviously, that it could bind complement proteins. We hypothesized that P-selectin could be such a protein, given that P-selectin expression in platelets is activation dependent (24). Furthermore, P-selectin has nine sushi domains (25), which are a common structural motif in many complement-binding proteins, such as CR1, CR2, and factor H (26, 27).

We found that P-selectin antibodies partially inhibited the binding of FITC-conjugated C3b to activated platelets, indicating that P-selectin is at least partly responsible for the binding of C3b to activated platelets. By establishing P-selectin as a specific C3b-binding protein, we showed that CHO-P bound severalfold more C3b than did control CHO cells. We obtained further evidence of a specific interaction between P-selectin and C3b by plasmon resonance binding, revealing that these two proteins indeed interact with each other with a K_d of 1 μM.

P-selectin not only bound C3b, but allowed the initiation and propagation of complement activation on the cell surface. CHO-P cells that were incubated in the plasma bound more C3 and generated significantly greater quantities of C3a than did the control cells. Also, CHO-P cells that were incubated in the plasma formed greater quantities of the C5b-9 complex on their surface and generated more C3a than control cells.

Collectively, our findings identify P-selectin as a C3b-binding protein that is sufficient for activating the complement system via the formation of the C3 convertase complex (C3bBb) on the surface of cells. We propose that, in the setting of P-selectin expression, the complement system becomes activated, leading to inflammation and tissue injury. The studies by Kyriakides et al. are consistent with this conjecture (28). These authors showed that soluble P-selectin inhibited complement-induced muscle injury in murine hindlimbs subjected to ischemia reperfusion. This inhibitory effect of soluble P-selectin appeared to depend on the interaction between P-selectin and complement proteins, and to require the sushi domains of P-selectin. Therefore, in light of the findings of our studies, we believe that in the experiments performed by Kyriakides and colleagues, the infused P-selectin competed for C3b binding, diminishing the amount of C3b deposited on the surfaces of cells.

In summary, we propose a model in which C3b generated by the spontaneous hydrolysis of C3 in the plasma, binds to P-selectin the surface of activated platelets (Fig. 7). C3b, together with factor B, forms the C3 convertase complex cleaving large quantities of C3 into C3a and C3b. C3a recruits leukocytes and induces inflammation, while C3b deposited on the surface of platelets leads to full activation of the complement system and the formation of C5b-9 complexes. Such a scheme would allow activation of the com-
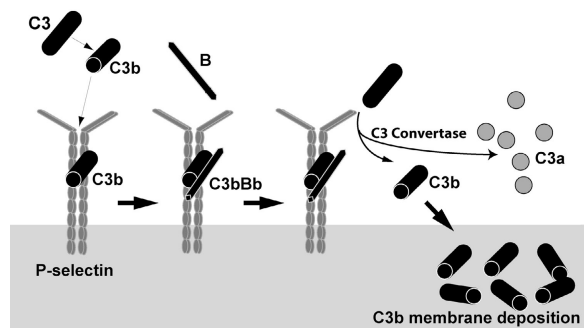
Figure 7. The proposed model P–selectin captures and immobilizes labile C3b. Binding of factor B to C3b initiates formation of C3 convertase (C3bBb) on the surface of activated platelet or endothelial cells. The C3 convertase complex cleaves C3 and mediates amplification of the complement activation (see Discussion for details).

plement system on activated platelets, thus inducing an inflammatory response localized to sites of thrombosis. Finally, although we specifically examined complement activation on the surface of platelets, we speculate that a similar phenomenon occurs on the surface of activated endothelial cells. If so, P-selectin may emerge as an important molecule for localizing complement activation at sites of thrombosis and inflammation.

MATERIALS AND METHODS

Platelet preparation. Blood was drawn from healthy individuals into a syringe containing 1/9 volume of 3.8% sodium citrate. PRP was obtained by centrifuging the blood at 700 g for 6 min. Platelet-poor plasma (PPP) was prepared by centrifuging PRP at 2000 g for 15 min. For some experiments, platelets were washed once in tris-buffered saline (TBS; 50 mM Tris–HCl and 0.9% NaCl, pH 7.4), in the presence of 5 ng/ml PGI$_2$ to avoid aggregation, and resuspended in Tyrode's buffer (137 mM NaCl, 1.2 mM Hepes, 2.7 mM KCl, 0.04 mM NaH$_2$PO$_4$ · H$_2$O, and 5.6 mM glucose, pH 7.4) containing 2 mM CaCl$_2$. The platelet count in PRP and washed platelet suspensions was adjusted to 250 × 10^3/μl using PPP and buffer, respectively. All the studies performed on blood samples were approved by the Institutional Review Board of Baylor College of Medicine.

Platelet activation. Platelets were activated with different agonists. For experiments in which platelets were activated with shear, 1 ml PRP was subjected to a shear rate of either 0, 1,000, or 10,000 s^{-1} for 1 min in a cone plate viscometer, prewarmed to 37°C (model RS1 visometer; Haake). In other experiments, platelets in plasma were activated with either 20 μM ADP (Sigma-Aldrich), 5 μM TRAP (Peninsula Laboratories), or 5 μg/ml type-1 collagen (Helena Laboratories), stirring continuously for 20 min at 37°C.

Cell lines. CHO-P cells (provided by W. Smith, Baylor College of Medicine, Houston, TX) were used to study the interaction between complement proteins and P-selectin. Mock-transfected CHO cells served as control cells. In the experiments assessing the effect of calcium and magnesium on binding of C3b to P-selectin, we used CHO-βIX cells (generated as in reference 14) as an additional negative control. The monocytic cell line, THP-1, was obtained from American Type Culture Collection.

Deposition of C3 on activated platelets and on heterologous cells. Deposition of C3 on platelets or CHO-P cells was measured by Western blotting and flow cytometry. For Western blotting, activated or resting plate-

lets from equal volumes of PRP were pelleted at 1,500 g for 10 min, washed with TBS buffer, lysed in 8% SDS-reducing sample buffer, and subjected to 7.5% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane, which was then blocked with nonfat milk (5 g/dl) and immunoblotted with a goat anti–human C3 polyclonal antibody (Advanced Research Technologies) and with an HRP-conjugated secondary antibody (Zymed Laboratories). We used Super signal Westpico chemiluminescent (Pierce Chemical Co.) substrate to detect proteins. As a loading control, the blotted membranes were treated with stripping buffer (Pierce Chemical Co.), and reprobed with mAbs against β-actin (Sigma-Aldrich), or GP Ibα (clone WM23; a gift from Michael C. Berndt, Monash University, Australia).

To study the binding of C3 to CHO cells, 10^5 cells (CHO-P or CHO) cells were incubated in 0.5 ml PPP for 30 min. Cells were washed once and were incubated with a 1:100 dilution of anti-C3 goat antiserum (Advanced Research Technologies) for 30 min. Cells were washed once, and were then incubated with FITC-conjugated secondary antibody (Zymed Laboratories). Samples were washed, and subsequently analyzed with a flow cytometer (model EPICS XL; Coulter). Background fluorescence was set using normal goat serum (Pierce Chemical Co.) instead of the anti-C3 antiserum.

Formation of C5b-9 complex on activated platelets or heterologous cells. The presence of membrane attack complexes (C5b-9) on platelets was studied by flow cytometry analysis using a rabbit polyclonal antibody against a neoepitope of the C5b-9 complex (Advanced Research Technologies). Approximately 10^6 TRAP-activated or resting platelets were incubated with saturating concentrations of the anti-C5b-9 antibody, and of a FITC-conjugated secondary antibody. Platelets were then diluted with 1 ml TBS and analyzed by flow cytometry. For experiments using heterologous cells, CHO-P or CHO cells were incubated in PPP for 60 min at 37°C, and studied with flow cytometry using anti–C5b-9 antibody, as just described for platelets.

Measurements of C3a. Formation of C3a was quantitated by BD OptEIA ELISA for human C3a (BD PharMingen). 1 ml PRP (platelet count, 250 × 10^3/μl) was stimulated with 20 μM ADP, 5 μM TRAP, or 5 μg/ml type-I collagen for 15 min at 37°C. Alternatively, 1 ml PRP was subjected to various shear rates for 1 min, as described above. Platelets not treated with any agonist and not subjected to shear served as the respective controls. Futhan, an inhibitor of complement activation, was added (for a final concentration of 50 μg/ml) to the samples after stimulation to prevent further C3a generation. In some experiments, neutrophils were isolated as described previously (29) and resuspended in autologous plasma. They were subsequently added to 100-μl aliquots of 2 × 10^5/μl of autologous platelets suspended in plasma to produce neutrophil counts ranging from 500 to 5 × 10^5 neutrophils/μl PRP, and which was subsequently stimulated with 30 μg/ml of type-1 collagen for 30 min.

For measuring C3a generation by CHO cells, 10^6 cells were incubated in 1 ml PPP for 60 min at 37°C. Futhan was added to the samples, the cells were sedimented, and the plasma supernatant was collected and stored at −80°C until assayed.

In other experiments, we measured C3a generation by CHO-P and CHO cells using a reconstituted complement system in the presence or absence of factor B. 1.5 × 10^6 cells were suspended for 60 min at 37°C in TBS with 1.5 mM CaCl$_2$ and 1 mM MgCl$_2$, supplemented with 600 μg/ml purified human C3, 500 ng/ml factor D, and with or without 112.5 μg/ml factor B. Cells were pelleted at 800 g for 5 min, the supernatant was collected and treated with 50 μg/ml futhan. C3a generation was measured by ELISA.

Binding of C3b to platelets and CHO cells. For assessing the binding of C3b to cells, purified C3b (Advanced Research Technologies) was labeled with FITC using FluoReproter protein labeling kit (Molecular Probes), according to the manufacturer's protocol. 1 million TRAP-activated washed platelets or 10^5 CHO-P and CHO cells were incubated with 2 μg/ml FITC-labeled C3b. Some experiments were performed in the

presence or absence of 2 mM CaCl$_2$ and 1 mM MgCl$_2$, with or without 5 mM EDTA. Cell-associated fluorescence (defined by an FS-SS gate) was measured with a flow cytometer.

Inhibition of C3b binding to activated platelet was investigated by incubating washed platelets with 5 μg/ml of rabbit polyclonal anti–P-selectin antibody (BD PharMingen) before adding FITC-conjugated C3b.

Cleavage of surface proteins on activated platelets. We cleaved surface proteins on activated platelets using protease K. Washed platelets were activated with 10 μM TRAP, as described before, and were then treated with 50 μg/ml protease K for 30 min, at room temperature. After washing the platelets three times in 10 ml TBS, binding of FITC-conjugated C3b was measured by flow cytometry, as described before.

Flow chamber studies. 30 μg/ml of purified C3b was immobilized onto glass coverslips by incubation at 37°C for 3 h. Coverslips were washed once with buffer, and blocked with 1% BSA (Sigma-Aldrich). Coated coverslips were mounted on the lower surface of a parallel plate flow chamber, as described previously (30). The flow chamber was then mounted onto an inverted stage microscope (model Eclipse TE300; Nikon) equipped with an image recording system. Platelets were activated with 5 μM TRAP for 10 min at 37°C, in the presence or absence of 25 μg/ml polyclonal anti–P-selectin antibody (BD PharMingen) or an anti-GPIbα antibody (clone WM23; a gift from M. Berndt, Monash University, Victoria, Australia), and were then perfused through the chamber at a flow rate of 0.2 ml/min, generating a wall shear stress of 2 dynes/cm^2. Albumin-coated coverslips were used as the controls.

Surface plasmon resonance binding assay. Surface plasmon resonance binding studies were performed using a BIAcore 2000 system as previously described (31), with minor modifications. Recombinant human P-selectin (50 μg/ml in 50 mM sodium acetate, pH 4.8; R&D Systems) was covalently coupled via amine coupling to a sensor chip (CM5) as directed by the manufacturer (BIAcore). The immobilization of the P-selectin resulted in ∼1,200 resonance units, corresponding to a surface density of ∼1 ng/mm^2. The binding assays were performed in PBS, with 1 mM CaCl$_2$ and 1 mM MgCl$_2$, pH 7.4 at 25°C with a flow rate of 10 μl/min. The binding of the C3b to the P-selectin–coated channel was corrected for nonspecific binding to the control channel (no protein; between 5 and 10%). P-Selectin binding at equilibrium was determined at several concentrations of C3b (0.5, 1.0, 3.0, and 5.0 μM). After measuring the C3b binding to P-selectin, the chip was regenerated by injection of 1 mM EDTA, 1 M NaCl, 0.1 M sodium citrate, pH 5.0, 10 mM deoxycholic acid, and 100 mM Tris, pH 9.0. The set of sensorgrams were best fit to the 1:1 Langmuir binding model with drifting baseline using the BIAevaluation software (version 3.0) provided by the manufacturer.

Statistics. All experiments were performed at least three times, each time using the blood from different donors. Data throughout the paper are presented as mean ± SD. Comparisons were made using Student's t test; $P < 0.05$ was considered to be statistically significant.

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