Thrombin generation in platelet-rich plasma (PRP) involves complex interactions between platelets and coagulation proteins. We previously reported that the addition of fibrin to PRP enhances tissue-factor initiated thrombin generation by ~40% and the current studies were designed to assess the mechanism(s) underlying thrombin generation in the absence and presence of fibrin. Blocking platelet GPIb/IIIa + αvβ3 receptors with a monoclonal antibody (MoAb) inhibited basal thrombin generation, but did not affect the enhancement produced by fibrin. In contrast, blocking GPIb with any of three different MoAbs had no effect on basal thrombin generation, but essentially eliminated fibrin enhancement of thrombin generation. When thrombin generation was tested in PRP deficient in von Willebrand factor (vWF), both basal and fibrin-enhanced thrombin generation were markedly reduced, and the addition of factor VIII did not normalize thrombin generation. Botrocetin, which induces the binding of vWF to GPIb, enhanced thrombin generation. In all studies, the ability of PRP to support thrombin generation correlated with the production of platelet-derived microparticles and serum platelet-derived procoagulant activity. Thus, two separate mechanisms, both of which depend on vWF, appear to contribute to platelet-derived procoagulant activity: one is independent of fibrin and relies primarily on GPIb/IIIa, but with a minor contribution from αvβ3; and the other is fibrin-dependent and relies on GPIb. These data may have implications for understanding the mechanisms of the abnormalities in serum prothrombin times reported in Bernard-Soulier syndrome, hemorrhage in von Willebrand disease (VWD), and the increased risk of thrombosis associated with elevated vWF levels.

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contains only traces of vWF (<2 ng per unit of factor VIII). Fluorescein isothiocyanate (FITC)-labeled annexin V (Apoptest, Oregon Green) was from NeXius Research BV (Hoeven, The Netherlands).

Murine MoAbs, 7E3 (anti-GPIb/IIa + αvβ3)9, 6D1 (anti-GPIb),9 and 6F1 (anti-GPIb/IIa)10 have been previously described in detail. MoAb CD42b (anti-GPIIb) was from Immunotech (Marseille, France) and MoAb AP-1 (anti-GPIb) was a kind gift of Dr Thomas Kunicki (Scirppus Institute, La Jolla, CA). R-phycoerythrin-conjugated MoAbs to human GPIIIa/IIa (5B12) and GPIb (AN51) were obtained from Dako (Glostrup, Denmark). An affinity-purified polyclonal antibody to vWF was obtained from the Central Laboratory of the Red Cross (CLB) in Amsterdam, The Netherlands.

Preparation of plasma. PRP was obtained by centrifuging fresh citrated blood (9 parts of blood to one part of 0.13 mol/L trisodium citrate) at 250g, 15°C for 10 minutes. The platelet count was adjusted to 3 × 10^9/mL using autologous platelet-poor plasma (PPP).

The study included four patients with Glanzmann thrombasthenia of Iraqi-Jewish descent (GPIIb/IIIa and αvβ3) and three of Arab descent (GPIb defect leading to loss of GPIIIa/IIa), but normal or increased vWF (residing in Israel).11 By courtesy of Dr Karly Hamulyak (Academic Hospital, Maastricht), blood was obtained from one patient with Glanzmann thrombasthenia, and one with von Willebrand disease (vWD) type IIa. By courtesy of Dr J. Eikelboom12 (Academic Hospital Leiden), blood was obtained from a patient with type III vWD (described in Weiss et al13).

Preparation of clots. Fibrin I clots (noncross-linked, des AA fibrin) were prepared as previously described using the snake venom protase Agital. Addition of clots to PPP did not cause coagulation within 2 hours and did not influence either the clotting time or thrombin generation in recalcified PPP. Three clots were added to PRP in the thrombin generation experiments, representing ~3 times the potential fibrin content of the PRP.

Measurement of thrombin generation. Thrombin generation in plasma was performed as described previously.14,15 In short, for thrombin generation in PPP, 20 µL of a kaolin suspension and 20 µL of Buffer A were added to 240 µL of PPP. At 4 minutes, 20 µL of PS/PC (20 µmol/L) was added and at 5 minutes, coagulation was triggered by adding 60 µL of 0.1 mol/L CaCl₂. For thrombin generation in PRP, 240 µL of PRP was incubated with 60 µL of Buffer A or buffer containing the antibody or other additions to be tested for 10 minutes at 37°C. Fibrin clots were added just before coagulation was initiated by adding 60 µL of 0.1 mol/L CaCl₂, 1.8 fmol/L tissue factor.

One minute after initiating coagulation, 10 µL-samples of the reaction mixture were taken at 1-minute intervals and added to prewarmed (37°C) cuvettes containing 490 µL of 200 µmol/L S2238 in buffer B. The reaction was stopped after 2 minutes by adding 300 µL of prewarmed (37°C) cuvettes containing 490 µL of 200 µmol/L S2238 in buffer B. 15°C for 10 minutes. The platelet count was adjusted to 10 8/mL using autologous platelet-poor plasma (PPP).

Measurement of residual prothrombin in serum. Residual prothrombin was assessed as previously described.7,15,18

RESULTS

The effects of adding fibrin clots to normal PRP and of blocking GPIIb/IIIa + αvβ3, GPIb, and GPIa/IIa (α2β1) receptors. Consistent with our earlier observations, adding fibrin clots to normal PRP enhanced the ETP by ~42%, peak thrombin generation by ~64%, platelet-derived microparticles (PDM) by ~44%, and PMPA by ~78%6 (Fig 1 and Table 1). It also decreased residual prothrombin by ~50% (Table 1). Also consistent with our earlier observations, blockade of GPIIb/IIIa + αvβ3 receptors by antibody 7E3 or the peptide d-RGDW (60 µmol/L) decreased ETP of PRP to 42% and 60% of normal, respectively5 (Fig 1 and Table 1). The other parameters of thrombin generation were affected in a manner consistent with the inhibitory effects of these agents on the ETP (Table 1). What was most remarkable, however, was the ability of fibrin clots to enhance thrombin generation in PRP in which GPIIb/IIIa + αvβ3 receptors on thrombin generation in the absence and presence of fibrin clots. Thrombin generation was triggered at t = 0 in PRP (adjusted to 3 × 10^9/mL) by recalcification and addition of tissue factor. (■) Control; (●) three fibrin clots added at t = 0; (+) PRP preincubated with antibody 7E3 (anti-GPIIb/IIIa + αvβ3); 20 µg/mL; (▲) preincubation with 7E3 and fibrin clots added.
αβ3 receptors had been blocked by 7E3 or d-RGDW (Fig 1 and Table 1).

Blocking the vWF binding domain of GPIb with antibody 6D1 had the mirror image effect of GPIIb/IIIa + αβ3 blockade; thus, it had no effect on thrombin generation in the absence of added fibrin, but prevented the procoagulant-enhancing effect of fibrin (Fig 2 and Table 1). Two other antibodies against GPIb (AP-1 and CD 42b) gave similar results (Table 1). When antibodies 7E3 and 6D1 were used in combination, the results were additive, with both a reduction in thrombin generation and near elimination of the enhancement of thrombin generation by adding fibrin (Table 1). The MoAb 6F1, which blocks GPIa/IIa, affected neither normal thrombin generation nor the enhanced thrombin generation in the presence of fibrin, and thus served as a control (Table 1). Ionomycin-treated PRP supported thrombin generation to the same extent as did normal PRP with added fibrin (Table 1). None of the antibodies inhibited thrombin generation supported by ionomycin-treated platelets (data not shown).

**Studies using PRP from patients with Glanzmann thrombasthenia.** Because antibody 7E3 blocks both platelet GPIIb/IIIa and αβ3, we tried to assess the contributions of each of these receptors by comparing the results using PRP from different patients with Glanzmann thrombasthenia. Iraqi-Jewish patients have no detectable GPIIb/IIIa or αβ3, whereas Israeli-Arab patients have virtually no GPIIb/IIIa, but approximately twice the normal level of platelet αβ3.11,19 In both patient groups, thrombin generation in PRP is decreased to about 60% of normal (Table 2). Antibody 7E3 decreased thrombin generation in PRP of two Arab patients tested (from 68% to 59% and from 35% to 25%), but had virtually no effect on thrombin generation in the PRP of Iraqi-Jewish patients (from 71% to 70% and from 63% to 65%) (Table 2). Addition of fibrin clots to the PRP of patients in either group resulted in increased thrombin generation (increases of 18%, 44%, and 103% for Arab patients B,
C, and D; and ≈28% for Iraqi-Jewish patient A), supporting the conclusions derived from the antibody studies, namely that the effect of fibrin does not require either GPIIb/IIIa or \( \alpha v \beta 3 \) receptors.

**Experiments with vWF.** Because vWF has been reported to bind to fibrin and support an interaction with platelet GPIb, we investigated the effect of vWF on thrombin generation. Decreasing vWF activity of normal PRP with a neutralizing antibody not only prevented the enhancement of thrombin generation produced by fibrin, but, unexpectedly it also diminished baseline thrombin generation (Fig 3). In contrast, thrombin generation in PPP was unaffected by vWF neutralization (Fig 3, inset), indicating that there was sufficient factor VIII coagulant activity in the antibody-treated plasma to support thrombin generation. Addition of ionomycin (Fig 3) or a frozen and thawed platelet lysate (not shown) restored thrombin generation, indicating that procoagulant phospholipids were indeed rate-limiting. To further assess whether the interaction of vWF and GPIb enhanced thrombin generation, we added botrocetin to PRP and found that it did, indeed, increase thrombin generation.

Table 2. Thrombin Generation in PRP of Normal and Glanzmann Thrombasthenia (GT) Patients

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Values are expressed as a percentage of a normal control assayed concomitantly. Legend as in Table 1.

Fig 3. Effect on thrombin generation of reducing vWF activity in plasma. (h) Control (normal PRP with 10 μg/mL rabbit IgG); (d) PRP preincubated with vWF antibody (10 μg/mL); (X) PRP preincubated with anti-vWF antibody (10 μg/mL), three fibrin clots added at t = 90 s; (A) preincubated with vWF antibody and 10 μmol/L ionomycin added at t = 10 s. (Inset) Thrombin generation in PPP. The reaction was triggered with PS/PC and Ca²⁺. (h) Control PPP with 10 μg/mL rabbit IgG; (X) PPP preincubated with anti-vWF (10 μg/mL).

Fig 4. Thrombin generation in PRP of a patient with mild type IIa vWF deficiency. The patient’s plasma contained 30% factor VIII and ≈4% of vWF antigen. (h) Control PRP (with 10 μg/mL rabbit IgG); X, patient’s PRP (rabbit IgG added); (●) patient’s PRP preincubated with anti-vWF antibody (10 μg/mL). Inset: thrombin generation in PPP (h) Normal control; (X) patient.
DISCUSSION

We previously observed two different phenomena related to platelets and thrombin generation: (1) in a fibrin-free system or in a system in which fibrin is generated late in the reaction, blockade of GPIIb/IIIa and to a lesser extent αvβ3 decreases thrombin generation, and (2) adding fibrin to PRP enhances thrombin generation. The present studies were designed to obtain data on the receptors, ligand, and mechanisms responsible for these phenomena. Despite the ability of GPIIb/IIIa to bind fibrinogen, polymerizing fibrin, and clotted fibrin under static or flow conditions,21-27 our current data indicate that the enhancing effect of fibrin on thrombin generation cannot be attributed to a GPIIb/IIIa-mediated mechanism because fibrin retains its stimulating effect in the presence of a GPIIb/IIIa + αvβ3 blocking antibody or peptide, as well as when added to the PRP of Glanzmann patients.

We next studied the interaction between fibrin-bound vWF and platelet GPIb.20 Although anti-GPIb MoAbs had no effect on thrombin generation in the absence of added fibrin clots, they essentially abolished the enhancing effect of the added fibrin. Thus, the fibrin effect seems to be mediated via GPIb. This observation provides a possible explanation for the abnormal prothrombin consumption previously reported in patients with Bernard-Soulier syndrome, whose platelets lack GPIIb28-32 and the abnormal prothrombin consumption that we previously reported when antibody 6D1 was added to normal blood.9

In view of the important role of GPIb in the fibrin-dependent enhancement of thrombin generation in PRP, it is perhaps surprising that in normal, recalcified PRP, thrombin generation initiated by tissue factor is minimally inhibited by blocking GPIb. The most likely explanation is that in these experiments, fibrin begins to form late in the process, at the very beginning of the thrombin burst, and thus there is insufficient time for it to affect the process. When fibrin in the form of preformed clots is
added before the thrombin burst occurs, it enhances thrombin generation and shortens the lag-phase in a GPIb-dependent mechanism. Taken together, our previous and current data indicate that these are two different pathways for augmenting platelet coagulant activity: (1) a GPIb/IIIa- and perhaps αβ3-dependent pathway that operates independently of fibrin, and (2) a fibrin-and GPIb-dependent pathway. Of note, based on data using neutralizing antibody to vWF and plasma of patients with vWD, as well as on our data using botrocetin, both pathways appear to depend on vWF, suggesting that vWF binding to GPIIb/IIIa (and perhaps αβ3) is important in the development of platelet coagulant activity.

The generation of PMPA and platelet-derived microparticles in serum and the consumption of prothrombin followed the same pattern of inhibition as did thrombin generation. This suggests that microparticle formation is responsible for the PMPA and that GPIIb/IIIa (and perhaps αβ3), GPIb, and vWF are all required for maximal microparticle formation in a fibrin-containing system. The ability of ionomycin-treated platelets and platelet lysates to overcome the abnormalities produced by the antibodies further suggests that the defects result in decreased microparticle formation.

From our results it appears that the fibrin in a clot or thrombus is not merely an inert, mechanical component. It also is clear that vWF, apart from its established function in platelet adherence and as a carrier of factor VIII, may also play an important role in the generation of thrombin through its effect on the generation of platelet microparticles and platelet coagulant activity. Because it has been proposed that the thrombosis associated with heparin-induced thrombocytopenia is linked to platelet microparticle formation, it is interesting to speculate that variations in vWF levels may account for the interindividual differences in thrombotic risk. Recently, platelet microparticles were found to support transcellular metabolism of eicosanoids, leading to activation of platelets and endothelial cells, as well as modulation of monocyte-endothelial interactions, and so it is possible that the mechanisms we are studying have implications for these phenomena as well. Finally, our observations have potential implications for understanding better the pathophysiology of the bleeding in vWD, as well as the association between elevated plasma vWF activity and acute myocardial infarction, as well as death after stroke.

ACKNOWLEDGMENT

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Fibrin-Dependent Platelet Procoagulant Activity Requires GPIb Receptors and von Willebrand Factor

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