RAPID COMMUNICATION

Platelets Inhibit Fibrinolysis In Vitro by Both Plasminogen Activator Inhibitor-1–Dependent and –Independent Mechanisms

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Platelet-rich thrombi are resistant to lysis by tissue-type plasminogen activator (t-PA). Although platelet α-granules contain plasminogen activator inhibitor-1 (PAI-1), a fast-acting inhibitor of t-PA, the contribution of PAI-1 to the antifibrinolytic effect of platelets has remained a subject of controversy. We recently reported a patient with a homozygous mutation within the PAI-1 gene that results in complete loss of PAI-1 expression. Platelets from this individual constitute a unique reagent with which to probe the role of platelet PAI-1 in the regulation of fibrinolysis. The effects of PAI-1–deficient platelets were compared with those of normal platelets in an in vitro clot lysis assay. Although the incorporation of PAI-1–deficient platelets into clots resulted in a moderate inhibition of t-PA–mediated fibrinolysis, normal platelets markedly inhibited clot lysis under the same conditions. However, no difference between PAI-1–deficient platelets and platelets with normal PAI-1 content was observed when streptokinase or a PAI-1–resistant t-PA mutant were used to initiate fibrinolysis. In addition, PAI-1–resistant t-PA was significantly more efficient in lysing clots containing normal platelets than wild-type t-PA. We conclude that platelets inhibit t-PA–mediated fibrinolysis by both PAI-1–dependent and PAI-1–independent mechanisms. These results have important implications for the role of PAI-1 in the resistance of platelet-rich thrombi to lysis in vivo.

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within exon 4 of the PAI-1 gene. This mutation results in the synthesis of an unstable PAI-1 mRNA and a truncated PAI-1 protein that lacks the reactive center of the molecule. Platelets from this individual contain no detectable PAI-1, but are otherwise normal. Hence, they constitute a unique reagent to probe the contribution of PAI-1 to platelet-mediated clot lysis resistance. We have compared the effects of PAI-1-deficient platelets with those of normal platelets in an in vitro clot lysis assay in which fibrinolysis was initiated by wild-type t-PA, streptokinase, or a mutant t-PA that is resistant to inhibition by PAI-1. The results of these studies suggest that platelets inhibit fibrinolysis by both PAI-1-dependent and PAI-1-independent mechanisms.

MATERIALS AND METHODS

Materials. Recombinant human t-PA (single chain; specific activity, 580,000 IU/mg) was obtained from Genentech (South San Francisco, CA). A recombinant human single-chain t-PA mutant (Lys296, Arg298, Arg299→Glu) that activates plasminogen normally, but which is highly resistant to inhibition by PAI-1 (inhibition rate constant 5.0×10^−6 mol/L·s^−1 vs. 1.4×10^−6 mol/L·s^−1 for wild-type t-PA) was prepared as previously described. The specific activity of mutant t-PA (determined by comparison to wild-type t-PA in the clot lysis assay described below) was approximately 250,000 IU/mg. Streptokinase (specific activity, approximately 3,000 IU/mg) and human Glu-plasminogen were from American Diagnostica (Greenwich, CT). Fibrinogen (prepared from human plasma by β-alanine precipitation and lysine-sepharose chromatography) was the gift of M. Naski. Prostaglandin E1, apyrase, human thrombin, and fluorescein isothiocyanate were from Sigma (St Louis, MO).

Platelet preparations. Blood samples were collected from an individual with homozygous PAI-1 deficiency, an individual heterozygous for the PAI-1 null allele (father of the homozygous individual), and from normal controls. The clinical characteristics and molecular genetic analysis of this PAI-1-deficiency pedigree have been reported previously. The individual with homozygous PAI-1 deficiency (proband) was a 10-year-old female with a congenital bleeding disorder of moderate severity. Routine indices of hemostasis were normal, but which is highly resistant to inhibition by PAI-1 (inhibi-

RESULTS

Effect of platelets on t-PA-mediated clot lysis. To determine the effect of normal platelets on fibrinolysis in this experimental system, fibrin clots were formed in the presence or absence of platelets and the rates of wild-type t-PA-mediated clot lysis were compared. The concentration of t-PA in these experiments was 0.67 U/mL. In the absence of platelets, clot lysis was greater than 90% complete within 120 minutes at 37°C (Fig 1). However, in the presence of normal platelets, clot lysis was markedly inhibited, with less than 1% clot lysis occurring by 120 minutes. Essentially identical results (<1% clot lysis by 120 minutes in the presence of platelets) were obtained when this experiment was repeated with platelets obtained from three other normal individuals.

The effects on clot lysis of washed platelets obtained from the individual with homozygous PAI-1 deficiency were compared with those of platelets from a normal control (Fig 2). Whereas essentially no lysis occurred by 120 minutes in the presence of normal platelets, clot lysis was significantly greater in the presence of washed platelets from the proband.
The effects of platelet concentration on t-PA-mediated clot lysis were also examined. Clot lysis was allowed to occur for 60 minutes in the presence of t-PA (0.67 U/mL) and platelets (0 to 2.4 \times 10^9/mL). For both normal and homozygous PAI-1-deficient platelets, the extent of clot lysis was inversely proportional to platelet concentration, although more extensive lysis consistently was observed in the presence of PAI-1-deficient platelets (Fig 3). Hence, PAI-1-deficient platelets inhibited clot lysis less than normal platelets. However, platelets lacking PAI-1 retain the capacity to inhibit fibrinolysis, and do so in a concentration-dependent manner. Of note, clot retraction was observed in this experimental system, and the extent of clot retraction (estimated by visual inspection) appeared proportional to platelet concentration.

Effect of platelets on mutant t-PA- and streptokinase-mediated clot lysis. To further assess the role of platelet PAI-1 in regulating clot lysis, studies similar to those described above were performed, except that PAI-1-insensitive plasminogen activators (ie, t-PA_{\text{Lys-296,Arg-298,Arg299→Glu}} or streptokinase) were substituted for wild-type t-PA. In these experiments, the effects of platelets from the PAI-1-deficient individual were compared with those of her father's platelets. Platelet-rich clots were formed in the presence of mutant t-PA (1.5 U/mL) or streptokinase (8 mU/mL) and the rates of clot lysis were determined. In contrast to experiments with wild-type t-PA, clots containing PAI-1-deficient platelets (obtained from the proband) and those containing platelets with normal PAI-1 content (obtained from the proband's father) lysed at similar rates when t-PA_{\text{Lys-296,Arg-298,Arg299→Glu}} (Fig 4A) or streptokinase (Fig 4B) were used to initiate plasmin formation.

Comparison of wild-type and mutant t-PA in the lysis of platelet-rich clots. If platelet PAI-1 contributes to platelet-mediated clot lysis resistance, then a PAI-1-insensitive t-PA should lyse platelet-rich clots more efficiently than wild-type t-PA. To test this hypothesis, either wild-type or mutant t-PA was used to lyse clots formed in the presence or absence of normal platelets. In preparation for these experiments, standard curves of t-PA concentration (both for wild-type and mutant enzymes) versus the percentage of clot lysis at 1 hour were constructed (data not shown) to allow selection of enzyme concentrations that resulted in essentially equal amounts of clot lysis in the absence of platelets (ie, 0.6 U/mL wild-type t-PA and 2.0 ng/mL PAI-1-resistant t-PA). As shown in Fig 5, using these concentrations of t-PA, similar rates of clot lysis were observed in the absence of platelets. However, in the presence of platelets (2.5 \times 10^9/mL), the same concentrations of wild-type or mutant t-PA resulted in significantly different rates of clot lysis. Whereas normal platelets markedly inhibited the fibrinolytic capacity of wild-type t-PA, the fibrinolytic activity of PAI-1-resistant t-PA was minimally inhibited under these same conditions.

DISCUSSION

Several results from these in vitro experiments suggest that PAI-1 is an important determinant of the antifibrinolytic effect of platelets. First, PAI-1-deficient platelets inhibited t-PA-mediated clot lysis to a substantially lesser extent than normal platelets. Second, the relative inhibition of fi-
brinolysis by platelets with normal PAI-1 content (compared with PAI-1-deficient platelets) was not observed when plasmin formation was initiated by PAI-1-insensitive plasminogen activators (ie, the PAI-1-resistant t-PA mutant or streptokinase). And finally, PAI-1-resistant t-PA was significantly more effective than wild-type t-PA in lysing clots containing normal platelets. However, these experiments also suggest that platelets inhibit fibrinolysis by a mechanism(s) that does not involve PAI-1. Although reduced compared with normal platelets, platelets lacking PAI-1 retained the capacity to inhibit fibrinolysis. These findings are consistent with a contribution of platelet-mediated clot retraction to lysis resistance, as suggested by Kunitada et al. Indeed, visible clot retraction was observed during our experiments and appeared to be proportional to platelet concentration for both normal and PAI-1-deficient platelets.

The results of our studies are also consistent with the report of Levi et al., in which an anti–PAI-1 monoclonal antibody markedly accelerated the capacity of t-PA to lyse platelet-rich clots in vitro. These investigators also showed that this antibody, which inhibits the interaction of PAI-1 with t-PA, enhances spontaneous clot lysis and inhibits thrombus extension in vivo. Braaten et al. also showed that an anti–PAI-1 monoclonal antibody accelerates clot lysis in vitro. In addition, this group used immuno-electron microscopy to demonstrate that PAI-1 in platelet-rich thrombi is localized on fibrin, as opposed to the platelet surface, and that PAI-1 is particularly abundant on fibrin fibers that are closely associated with the platelet surface. These investigators hypothesize that the binding of PAI-1 to platelet-associated fibrin renders it more resistant to lysis, because fibrin-bound PAI-1 retains the capacity to inhibit t-PA. In contrast, in studies using pharmacologic concentrations of t-PA, Kunitada et al. concluded that PAI-1 is not a determinant of platelet-mediated clot lysis inhibition. These investigators observed that a PAI-1-resistant t-PA mutant at a concentration of 800 U/mL did not lyse platelet-rich clots faster than wild-type enzyme. In addition, no differences were observed in the t-PA activity of supernatants of clots prepared from platelet-poor versus platelet-rich plasma. The enhanced efficiency of a PAI-1-resistant t-PA (compared with wild-type enzyme) in our experimental system, but lack thereof in the experiments of Kunitada et al., is most likely explained by the substantial differences in t-PA concentrations used in the two studies (approximately 1,000-fold). Platelets contain approximately 4,000 molecules of PAI-1 per cell. Therefore, in clot lysis experiments using a platelet concentration of 3 x 10^6/mL and a t-PA (specific activity, 580,000 U/mg) concentration of 800 U/mL (ie, the conditions of Kunitada et al.), the molar ratio of t-PA:PAI-1 is 10:1. In addition, because as much as 95% of platelet PAI-1 is reported to exist in a latent, or inactive, form, the ratio of t-PA to functional inhibitor may have exceeded 100-fold in these experiments. Under these conditions, it is probably not possible to observe a PAI-1 effect and, hence, to discriminate between the lytic efficiencies of wild-type and PAI-1-resistant enzymes. Taken together, these studies suggest that clot-associated PAI-1 may inhibit endogenously mediated fibrinolysis, in which local t-PA concentrations are probably in the nanogram per milliliter range, but not pharmacologically mediated fibrinolysis, in which plasma t-PA concentrations can exceed 1 µg/mL.
PLATELET PAI-1 AND FIBRINOLYSIS

However, the concentration of platelets typically used in vitro clot lysis assays (10^9 to 10^9/mL) is probably considerably less than the concentration of platelets within a hemostatic plug or platelet-rich thrombus. For example, in our experimental system, platelets constituted less than 1% of the total volume of the clot lysis mixture. However, microscopic evaluation of coronary thrombi retrieved from patients with acute myocardial infarction or sudden cardiac death shows that they consist, to a considerable extent, of essentially solid masses of platelets with interspersed islands of fibrin. Hence, the concentration of PAI-1 in platelet-rich clots in vivo may be considerably higher than is present in vitro assays. Consistent with this hypothesis, Potter van Loon et al. showed that the average amount of PAI-1 antigen in human arterial thrombosis, which are frequently platelet-rich, is 150 times that detected in an equivalent volume of platelet-poor plasma. Similarly, the mean concentration of PAI-1 in experimentally induced porcine platelet-rich thrombi (36 µg/mL) exceeds that detected in plasma by greater than two orders of magnitude. Whether active PAI-1 accumulates in human platelet-rich thrombi in sufficient concentrations to inhibit pharmacologically-induced fibrinolysis is unknown. However, platelets contain vitronectin, which stabilizes PAI-1 in the active conformation, and phospholipid vesicles can convert latent PAI-1 to the active form, suggesting that reactivation of latent PAI-1 may occur on cell surfaces in vivo.

In summary, our experiments with PAI-1-deficient platelets and plasminogen activators with differential sensitivity to inhibition by PAI-1 suggest that PAI-1 is an important determinant of platelet-dependent clot lysis inhibition in vitro. Our studies also suggest that platelets inhibit fibrinolysis in a PAI-1-independent manner, consistent with prior observations that platelet-mediated clot retraction inhibits fibrinolysis. Although these results suggest that platelet PAI-1 may play an important role in the regulation of fibrinolysis within the microenvironment of the platelet-rich clot, additional studies are necessary to adequately test this hypothesis. For example, animal studies comparing the lysis of platelet-rich thrombi in response to wild-type versus PAI-1-resistant t-PA's should help to define the role of PAI-1 in platelet-mediated clot lysis inhibition in vivo. In addition to defining mechanisms underlying the inhibitory effect of platelets on fibrinolysis, such studies may also suggest alternative strategies for enhancing thrombolytic therapy in patients with acute thrombotic disease.

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