Plasminogen Activator Inhibitor–1 Released From Activated Platelets Plays a Key Role in Thrombolysis Resistance

Studies With Thrombi Generated in the Chandler Loop

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Abstract To investigate the potential role of plasminogen activator inhibitor–1 (PAI-1), which is released from the α-granules of activated platelets, in thrombolysis resistance, we employed a model (the "Chandler loop") that mimics the formation of arterial thrombi in vivo and that can be manipulated in terms of rheological parameters and composition of blood cells. Light and electron microscopy revealed that the distribution of blood cells in Chandler thrombi is polarized, as it is in arterial thrombi, resulting in platelet-rich "white heads" and red blood cell-rich "red tails." Resistance toward tissue-type plasminogen activator (TPA)–mediated thrombolysis parallels the presence of platelets that are fully activated in this system. We demonstrate that the PAI-1 released by the α-granules is preferentially retained within the thrombus and that the concentration of PAI-1 antigen is higher in the head than in the tail of the thrombus. The relative thrombolysis resistance of the heads of Chandler thrombi can be largely abolished by inclusion of an anti–PAI-1 monoclonal antibody that blocks that inhibitory activity of PAI-1 toward TPA. We propose that PAI-1, released from activated platelets, plays a key role in thrombolysis resistance and/or reocclusion after thrombolytic therapy. This is due to binding of PAI-1 to polymerized fibrin within the thrombus, followed by inhibition of TPA-mediated fibrinolysis. (Arterioscler Thromb. 1994;14:1452–1458.)

Keywords • PAI-1 • thrombolysis resistance • platelets • fibrinolysis • Chandler loop

The significance of plasminogen activator inhibitor–1 (PAI-1) as an essential regulatory protein of the fibrinolytic system has recently received substantial support. Ginsburg and coworkers report that the bleeding disorder of a 9-year-old Amish girl is due to a homozygous genetic defect in the PAI-1 gene that results in the absence of functional PAI-1 protein and, consequently, in excessive fibrinolysis. In vivo, PAI-1 is synthesized by endothelial cells and by megakaryocytes. After synthesis in the latter cell type, PAI-1 is presumably sequestered in platelets and stored in the α-granules. Platelets constitute the main reservoir of PAI-1 in blood, containing 4000 to 8000 molecules per cell, whereas the concentration of the protein in plasma is extremely low. From these data, it can be deduced that activation of platelets and release of the contents of the α-granules would result in a high local PAI-1 concentration in thrombi (up to 0.2 μmol/L). Based on the vast difference in concentration between PAI-1 in these compartments, we propose that platelet PAI-1 is the relevant regulatory protein for the fibrinolytic system. Support for this view stems both from in vitro and in vivo experiments. First, we reported that tissue-type plasminogen activator (TPA)–mediated fibrinolysis can be fully inhibited by (activated) platelets in a purified system, consisting of: Glu-plasminogen, 125I-labeled fibrinogen, and thrombin. Furthermore, in the presence of monoclonal antibodies, which block the activity of PAI-1, the inhibitory effect of activated platelets could be completely abolished. Second, the inclusion of such anti–PAI-1 monoclonal antibodies in thrombi, which were generated under static conditions in the jugular vein of the rabbit, promoted endogenous thrombolysis and partially prevented extension of the thrombus. But neither model incorporates the effect of the rheology of flowing blood on the cellular composition and the morphology of generated thrombi and, possibly, on the lysis of fibrin. Specifically, evidence has been obtained showing that arterial, platelet-rich thrombi are particularly resistant toward thrombolysis and that the phenomenon of reocclusion after thrombolytic therapy is associated with platelet-rich thrombi.

In this article we assess the notion that platelet PAI-1 is fixed within a thrombus and is responsible for thrombolysis resistance. The rationale for this view stems from in vitro experiments demonstrating that purified PAI-1 specifically binds to polymerized fibrin. Moreover, PAI-1 bound to fibrin effectively forms inactive complexes with TPA and thus limits TPA-mediated fibrinolysis. These observations indicate that PAI-1 released from platelets may be retained within thrombi and, consequently, may be a major cause for thrombolysis resistance. To study the role of platelet PAI-1, we
required a system that could be manipulated with regard to the composition of thrombi while simultaneously incorporating the consequences of the rheology of flowing blood. In 1958, Chandler developed a device (the "Chandler loop") that generates thrombi that mimic those formed under arterial flow conditions. A further validation of this model is reported here. We analyzed the morphology of Chandler thrombi by light and electron microscopy and found a similar polarized distribution of platelets and red blood cells as in arterial thrombi formed in vivo. This feature results in platelet-rich upstream sections ("white heads") that are relatively resistant to TPA-mediated thrombolysis in contrast to the red blood cell–rich downstream parts ("red tails"). Thus, we consider the Chandler loop a useful model with which to study the effect of rheology on the composition of thrombi and, specifically, to study thrombolysis resistance. We provide further evidence for an important role of PAI-1 in the control of fibrinolysis and indicate that thrombolysis resistance of platelet-rich thrombi is largely due to the presence of PAI-1 released from the α-granules and subsequently fixed within thrombi by binding to polymerized fibrin.

Methods

Materials

Radioactive materials and a radioimmunoassay for the determination of β-thromboglobulin (β-TG) were purchased from the Radiochemical Centre. Actilys TPA was purchased from Boehringer Ingelheim. Human fibrinogen grade L was obtained from KabiVitrum. The material was treated with 1 mmol/L diisopropyl phosphorofluoridate and with lysinopeptide to inactivate potentially contaminating serine proteases and to remove traces of plasminogen, respectively. 125I-Labeled fibrinogen was prepared by using the chloramine-T method. Fibrinogen clotability after labeling was ≥95%. Glu-plasminogen was purified from fresh-frozen human plasma. Human thrombin (2920 National Institutes of Health units per milligram protein) was purchased from Sigma Chemical Co. Streptokinase was obtained from Calbiochem, and azuro II/methylene blue was purchased from Merck AG.

Chandler Thrombi

Blood was obtained from healthy volunteers and collected in 1/8 volume of 15.6 mmol/L citric acid, 89.4 mmol/L trisodium citrate, 15.9 mmol/L sodium biphosphate, 161 mmol/L glucose, and 1.6 mmol/L adenosine. Platelet-rich and platelet-poor plasma were prepared by centrifuging blood at room temperature for 10 minutes at 380g and for 10 minutes at 1000g, respectively. Concentrates of red blood cells were prepared by washing the pellet of platelet-poor plasma twice with phosphate-buffered saline (PBS), 2% (wt/vol) glucose, and finally with plasma of the corresponding blood type. Care was taken to completely remove any huffy coat that arose after centrifugation. Reconstituted whole blood was prepared by adding the concentrate of red blood cells and platelet-rich plasma to platelet-poor plasma, yielding a hematocrit of 45. Chandler thrombi were produced as described before with minor modifications. Briefly, 2 mL blood was introduced into polyvinylchloride tubing (length, 40 cm; internal diameter, 3.2 mm), after which the blood was recalcified by adding 36 μL of 1 mol/L CaCl₂. Coagulation was initiated by adding 20 μL of 0.05 U/μL human thrombin. The tube was immediately closed to form a circle ("loop") and placed on a 45°-tilted turntable that was rotated at 12 rpm. After rotation for 30 minutes at room temperature, the thrombus was decanted from the loop and briefly washed with PBS to remove adhering serum.

Light and Electron Microscopy

Chandler thrombi were produced as described above. After briefly washing with PBS, the thrombi were fixed in a mixture of 2% (vol/vol) paraformaldehyde and 2.5% (vol/vol) glutaraldehyde in 0.1 mol/L sodium cacodylate (pH 7.4). Subsequently, the fixed thrombi were embedded in epoxy resin (Epon 812), and semithin sections were cut from the head and tail of the thrombus and stained with azuro II/methylene blue.

For electron microscopy ultrathin sections were stained with uranyl acetate and Reynolds' lead citrate and viewed in a JEOL 1200 CX electron microscope.

Platelet Activation Assay

Concentrations of β-TG were determined in platelet releases, in sera of static whole blood clots, and in sera of Chandler thrombi. Purified platelets were diluted to a concentration of 2×10⁸ per milliliter in Tyrode's buffer. Platelet activation was achieved by adding various concentrations of thrombin either to 100 μL of a suspension of purified platelets or to whole blood, respectively, followed by an incubation for 5 minutes at 37°C. The mixture was cooled on ice for 1 minute and was then centrifuged for 10 minutes at 15 000g. The concentration of β-TG was measured in the platelet release, in the supernatant of whole blood, and in the serum of Chandler thrombi, respectively, by using the β-TG radioimmunoassay according to the manufacturer's instructions.

Determination of PAI-1 Antigen

Whole Chandler thrombi or separated heads/tails were disintegrated either by sonication or by the administration of additional Glu-plasminogen and streptokinase (2 μmol/L and 200 IU/mL, respectively; total volume, 500 μL). The amount of PAI-1 in these thrombi and the corresponding sera was determined by an immunoradiometric assay that relied on two different monoclonal antibodies against PAI-1.

Thrombolysis of Chandler Thrombi

Citrated blood was supplemented with 5 μL 125I-labeled fibrinogen (9.2 PBq/mol protein, 5.88 mol/L) prior to recalcification and coagulation to monitor the time-dependent release of fibrin degradation products from plasmin-digested thrombi. After generation in the Chandler loop, the thrombi were briefly washed with PBS to remove adhering serum and were subsequently cut transversely to separate the upstream part (the head) from the downstream part (the tail). The radioactivity of these segments was determined separately in a gamma counter to establish the initial amount of radioactivity in the thrombus. The heads and tails were separately subjected to thrombolysis by adding 300 μL PBS containing 2 μmol/L Glu-plasminogen and the indicated concentrations of TPA, followed by an incubation at 37°C for various periods of time. Aliquots of 10 μL were obtained at the indicated time points, and the radioactivity was determined. The measured radioactivity was correlated to the input and expressed as the percentage of clot lysis. In some experiments varying concentrations of monoclonal antibodies were added to the blood before the initiation of coagulation.

Results

Morphology of Chandler Thrombi as Analyzed by Light and Electron Microscopy

The rheology of flowing blood is a major determinant of the composition and morphology of thrombi and of great influence on the efficiency of thrombolysis. Specifically in arteries, the rheological circumstances result in a polarized disposition of blood cells within thrombi. The upstream part contains a high concentration of platelets and few red blood cells ("white head"), whereas most of the red blood cells and relatively few
platelets are assembled in the downstream section ("red tail"). To investigate thrombolysis resistance, we used an in vitro model designated as the Chandler loop. We analyzed the morphology of thrombi produced in the Chandler loop under "arterial" flow conditions and compared their characteristics with defined features of arterial thrombi formed in vivo. To that end, serial transverse sections were prepared and analyzed either by light or electron microscopy (Figs 1A, 1B, and 2). Light microscopy clearly showed that virtually all the platelets are encountered in large aggregates that are assembled in the heads (Fig 1B, arrowheads). Electron microscopy (Fig 2) showed that most platelets contained only mitochondria, indicating that platelet degranulation had occurred. An occasional platelet still contained α-granules. A polarized distribution of leukocytes and red blood cells is observed in these thrombi. Leukocytes are present predominantly in the heads of the thrombi, whereas red blood cells are predominantly present in the tails. From these observations we conclude that the morphology of Chandler thrombi is similar to that of the genuine arterial thrombi encountered in vivo.

Activation of Platelets in Thrombi Formed in the Chandler Loop

We determined the extent of platelet activation after coagulation of whole blood in the Chandler loop by measuring the serum concentration of the α-granule-specific protein β-TG. For that purpose, the total content of releasable β-TG per defined number of platelets was estimated by activating either purified platelets or whole blood with different thrombin concentrations (Fig 3). The optimal amount of β-TG released by either 2×10⁶ purified platelets or 2×10⁷ platelets contained in whole blood was 390±47 pmol (mean±SEM; n=10). This value agrees well with previously reported data on the release of β-TG from platelets. Similarly, the amount of β-TG detected in the sera of thrombi generated from whole blood in the Chandler loop was 386±8.3 pmol (mean±SEM; n=4) β-TG per 2×10⁷ platelets. Consequently, we conclude that the Chandler loop system, using whole blood as a source to generate arterial-like thrombi, allows full activation of platelets and complete release of their α-granule contents. Hence, it is expected that yet another α-granule-specific protein, PAI-1, the physiological regulator of the fibrinolytic system, will be liberated from activated platelets in Chandler thrombi as well.

Retention of PAI-1 by Thrombi Produced in the Chandler Loop

We used thrombi produced in the Chandler loop by reconstituting whole blood with an increasing number of
intact purified platelets (ranging from $2 \times 10^9$ to $1 \times 10^{10}$ per milliliter) to analyze the retention of PAI-1 due to binding to fibrin.\textsuperscript{12,13} To that end, the amount of PAI-1 antigen was determined both in plasin-digested Chandler thrombi and in the corresponding sera. After thrombin-induced coagulation and activation of reconstituted whole blood containing an increasing number of platelets, the concentration of PAI-1 in serum increased fivefold (from 2 to 10 nmol/L). The concentration of PAI-1 in the thrombus paralleled the number of administered platelets and increased approximately 50-fold, reaching a final concentration of 210 nmol/L at the highest platelet number. Furthermore, we separately measured PAI-1 antigen in plasin-digested heads and tails of Chandler thrombi produced from native whole blood. The heads contained 2 nmol/L PAI-1, whereas the PAI-1 content of the tails was below the detection limit of 0.2 nmol/L (n=5). Alternatively, we disintegrated heads and tails by sonification. The heads contained 5 nmol/L PAI-1 and the tails contained 1.5 nmol/L (n=4). These data demonstrate that PAI-1 is preferentially retained within the heads of Chandler thrombi. Based both on our previous observations using purified components\textsuperscript{12} and on the analysis of Chandler thrombi presented here, we tentatively conclude that PAI-1 is retained within arterial thrombi that arise in vivo under pathological conditions.

**Polarized Thrombolysis Resistance in Chandler Thrombi**

Our morphological analysis revealed a close similarity between Chandler thrombi and arterial thrombi formed in vivo, particularly with respect to the preferential occurrence of large platelet aggregates in the upstream sections (heads). In view of the preceding arguments, we speculated that the heads of arterial thrombi are relatively resistant to TPA-mediated thrombolysis due to the presence of PAI-1 released from the α-granules and fixed by the fibrin network. To test this possibility, thrombin produced in the Chandler loop and radiolabeled with $^{125}$I-fibrinogen were divided into a “head segment” and a “tail segment” that were separately subjected to thrombolysis by using doses of TPA (0 to 2 nmol/L) that correspond to at most 50-fold the endogenous concentration.\textsuperscript{23,24} The results (Fig 4, top) show that the heads are significantly more resistant to TPA-mediated thrombolysis than the tails. Tails digested for 180 minutes displayed 50% clot lysis at 0.9 nmol/L TPA, whereas an extrapolation to 50% clot lysis of the heads
Thrombolysis Resistance of the Heads of Arterial Thrombi Is Largely due to PAI-1–Mediated Inhibition of Fibrinolysis

The concurrency of polarized thrombolysis resistance, the presence of platelet aggregates, and the retention of platelet PAI-1 led us to postulate that PAI-1 is a major component in the process of thrombolysis resistance and/or reocclusion after treatment of patients suffering from acute myocardial infarction with thrombolytic agents. To substantiate this assumption, various amounts of a murine monoclonal anti-human PAI-1 antibody (CLB-2C8) were included during the formation of Chandler thrombi. This antibody fully blocks the inhibitory activity of PAI-1 toward TPA in vitro. Subsequently, we measured thrombolysis of both heads and tails of these thrombi by using 0.9 nmol/L TPA (Fig 5). The presence of the anti–PAI-1 monoclonal antibody CLB-2C8 clearly resulted in a distinct increase of clot lysis of the heads, whereas the antibody hardly affected clot lysis of the tail segments. As outlined before, these differences can be expressed as a clot-lysis efficiency ratio of heads versus tails after 3 hours of TPA-mediated thrombolysis. In the presence of the optimal concentration of the antibody CLB-2C8, this ratio increases from 0.6 to 0.9. A further increase of the CLB-2C8 concentration did not alter this ratio. From these observations we conclude that the resistance of platelet-rich heads of arterial thrombi toward TPA-mediated thrombolysis is largely due to inhibition of fibrinolysis by PAI-1 released from platelet α-granules and retained within the thrombus by specific binding to polymerized fibrin.

Discussion

The treatment of patients suffering from acute myocardial infarction is hampered by the occurrence of reocclusion after thrombolytic therapy. Reocclusion in experimental animals is associated with the presence of platelet-rich thrombi. Consequently, several adjunctive approaches are presently developed that aim either at the inhibition of thrombin activity or at the prevention of platelet activation and aggregation. In this study we explored an alternative approach, namely, inhibition of the major inhibitor of the fibrinolytic system, i.e., platelet PAI-1, to limit thrombolysis resistance. To investigate this option, we used an in vitro model (ie, the Chandler loop) that uses whole blood or reconstituted blood and

![Graph showing clot lysis as a function of tissue-type plasminogen activator (TPA) concentration and kinetics of clot lysis.](image)

![Graph showing effect of anti-plasminogen activator inhibitor-1 (PAI-1) monoclonal antibody (MoAb) CLB-2C8 on clot-lysis efficiency.](image)
that incorporates the profound influence of arterial rheology on the composition and morphology of thrombi.\textsuperscript{14,26} As shown here, Chandler thrombi are morphologically similar to arterial thrombi formed in vivo, in particular with regard to the polarized distribution of fully activated platelet aggregates and red blood cells. Therefore, we consider this model to be adequate for a study of the thrombolytic characteristics of arterial thrombi. Furthermore, data generated with the Chandler loop system can be used as guidelines to devise animal models for thrombosis.\textsuperscript{7,27,28}

The rationale for exploring prevention of the inhibition of the fibrinolytic system to avoid reocclusion stems from the following considerations. First, purified PAI-1 specifically binds to polymerized fibrin.\textsuperscript{12,13} These observations have been recently extended by Braaten and coworkers,\textsuperscript{29} who showed that PAI-1 released from activated platelets binds to fibrin fibers in the vicinity of platelet remnants. Second, the reactive center of PAI-1 is not involved in fibrin binding, since fibrin-bound PAI-1 is fully able to form inactive complexes with TPAs.\textsuperscript{6,12} Next, the inactive TPA/PAI-1 complexes dissociate from the fibrin matrix but subsequently rebinding to fibrin by virtue of the fibrin-binding “finger” and “kringle 2” domains of TPA.\textsuperscript{30} From these observations we postulated that the inactive TPA/PAI-1 complexes actually compete with active TPA for the same binding sites on fibrin, ultimately resulting in efficient inhibition of fibrinolysis. Third, the significance of an inhibitor to control the activity of a target serine protease is predominantly dictated by the rate of association and by the local concentration of both reactants.\textsuperscript{31} Although it has been well established that the second-order association rate constant between PAI-1 and TPA is relatively high (3 x 10$^7$ [mol L$^{-1}$ s$^{-1}$]),\textsuperscript{32,33} it should be taken into account that the endogenous plasma concentration of both components is extremely low (about 40 pmol/L TPA and 200 pmol/L PAI-1, respectively).\textsuperscript{25,24,24,33}

Based on these considerations, it is unlikely that plasma PAI-1 is an important regulator of the fibrinolytic system. By contrast, fixation of platelet PAI-1 by fibrin binding would provide a high local PAI-1 concentration (up to 0.2 pmol/L), and consequently efficient inhibition of TPA-mediated fibrinolysis would occur mainly because the affinity of TPA for PAI-1 greatly exceeds that of TPA for the obligatory, fibrinolytic stimulator fibrin.\textsuperscript{28} Hence, we conclude that platelet PAI-1 fulfills the requirements to act as a relevant inhibitor of TPA.

Platelets preferentially assembled in the heads of Chandler thrombi were activated and released the proteins that are stored in the $\alpha$-granules. Accordingly, PAI-1 is liberated from activated platelets but subsequently is predominantly retained within the thrombus by binding to fibrin.\textsuperscript{12,15,29} In addition, platelet PAI-1 released in the context of a generated thrombus is fully active and may limit TPA-mediated fibrinolysis.\textsuperscript{31} Here, this notion is supported by two sets of experimental results, namely, that the heads of Chandler thrombi are relatively resistant to thrombolysis compared with the tails. This observation agrees with the distribution of PAI-1 over the heads and the tails. Furthermore, the relative thrombolysis resistance of the heads can be relieved to a large extent by the inclusion of a monoclonal antibody (CLB-2C8) that blocks the inhibitory activity of PAI-1. However, it should be noted that the clot-lysis efficiency of the heads of Chandler thrombi in the presence of an optimal concentration of the antibody CLB-2C8 did not entirely reach the efficiency of clot lysis of the corresponding tails (increase of clot-lysis ratio from 0.6 to 0.9; tails were arbitrarily set at 1.0). Apparently, the remaining thrombolysis resistance of the heads is associated with the presence of platelets but is independent of the presence of PAI-1. We assume that the remaining resistance is due to the presence of $\alpha$-antiplasmin that is also stored in the $\alpha$-granules of platelets and that can be cross-linked to fibrin by platelet factor XIII.\textsuperscript{36,39} Indeed, the administration of antibodies directed against $\alpha$-antiplasmin has been shown to promote thrombolysis in vitro.\textsuperscript{40} In addition, evidence has been presented that factor XIII-dependent clot retraction may contribute to thrombolysis resistance as well.\textsuperscript{41} Finally, it has been proposed that clot-bound thrombin is protected from inactivation by heparin-antithrombin III and may contribute to thrombolysis resistance/reocclusion.\textsuperscript{42} Hence, from our data using the described model system, we deduce that thrombolysis resistance is largely due to the inhibition of TPA-mediated fibrinolysis by platelet PAI-1, whereas the remaining resistance is conceivably due to clot retraction and to the presence of $\alpha$-antiplasmin and clot-bound thrombin.

We consider the data reported in this article and our recent observations on the effect of an included anti-PAI-1 monoclonal antibody to promote thrombolysis and to limit thrombus growth in the rabbit jugular vein model\textsuperscript{7} as strong support for an important role of platelet PAI-1 in the control of thrombolysis. The data also provide support for the development of anti-PAI-1 antibodies, or fragments thereof, to serve as an adjunctive agent to prevent reocclusion after thrombolytic therapy.

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