Thrombin Neutralizes Plasminogen Activator Inhibitor 1 (PAI-1) That Is Complexed with Vitronectin in the Endothelial Cell Matrix

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Abstract. Vitronectin endows plasminogen activator inhibitor 1 (PAI-1), the fast-acting inhibitor of both tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), with additional thrombin inhibitory properties. In view of the apparent association between PAI-1 and vitronectin in the endothelial cell matrix (ECM), we analyzed the interaction between PAI-1 and thrombin in this environment. Upon incubating ^{125}I-labeled α-thrombin with endothelial cell matrix (ECM), the protease formed SDS-stable complexes exclusively with PAI-1, with subsequent release of these complexes into the supernatant. Vitronectin was required as a cofactor for the association between PAI-1 and thrombin in ECM. Metabolic labeling of endothelial cell proteins, followed by incubation of ECM with t-PA, u-PA, or thrombin, indicated that all three proteases depleted PAI-1 from ECM by complex formation and proteolytic cleavage. Proteolytically inactive thrombin as well as anticoagulant thrombin, i.e., thrombin in complex with its endothelial cell surface receptor thrombomodulin, did not neutralize PAI-1, emphasizing that the procoagulant moiety of thrombin is required for a functional interaction with PAI-1. A physiological implication of our findings may be related to the mutual neutralization of both PAI-1 and thrombin, providing a new link between plasminogen activation and the coagulation system. Evidence is provided that in ECM, procoagulant thrombin may promote plasminogen activator activity by inactivating PAI-1.

THE activation of plasminogen is a key step both in the fibrinolytic system and in various physiological and pathophysiological processes, involving extracellular matrix degradation (e.g., inflammatory reactions, malignant invasion and metastasis, ovulation, nidation, cell migration and tissue remodeling) (for review see Dano et al., 1985). Therefore, the precise regulation of plasminogen activation with respect to time and location is of critical importance for the organism. A major physiological mechanism for the control of plasminogen activation is provided by the action of plasminogen activator inhibitor 1 (PAI-1),[1] the fast-acting inhibitor of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) (for review see Schleef and Loskutoff, 1988). Recently, considerable support for the role of PAI-1 in prevention of tumor-mediated extracellular matrix degradation has been reported (Cajot et al., 1990). These investigators showed, using co-culture experiments, that a PAI-1–expressing cell could abrogate the invasive property of another cell type.

During the past few years, numerous studies have revealed important biochemical and cell biological data on PAI-1. Molecular cloning of full-length PAI-1 cDNA and elucidation of its nucleotide sequence has predicted the amino acid sequence of the protein. These studies showed that the inhibitor belongs to the serine protease inhibitor ("serpin") superfamily of proteins (Ny et al., 1986; Pannekoek et al., 1986; Ginsburg et al., 1986; Andreasen et al., 1986*b*). A serpin functions by acting as a pseudo-substrate and traps the enzyme by forming a 1:1 equimolar, SDS-resistant, inactive complex (Travis and Salvesen, 1983; Huber and Carrell, 1989). The relevance of a serpin-protease interaction is determined by (*a*) their appearance in the same compartment

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1. *Abbreviations used in this paper*: ECM, endothelial cell matrix; HUVEC, Human umbilical vein endothelial cell; PAI-1, plasminogen activator inhibitor 1; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.

or cell type, (*b*) their concentrations in that compartment, and (*c*) their association rate. The latter parameter may be affected by cofactors, such as glycosaminoglycans, that significantly accelerate the interaction between the serine protease thrombin and the serpins antithrombin III (Rosenberg and Damus, 1973), heparin cofactor II (Tollefsen and Blank, 1981), and protease nexin I (Baker et al., 1980).

PAI-1 is synthesized and secreted by cultured, vascular endothelial cells (Loskutoff and Edgington, 1977) and a large number of cell lines (e.g., the human fibrosarcoma cell line HT-1080 [Andreasen et al., 1986*a*]). Moreover, it has been reported that PAI-1 is deposited in the subcellular matrix of a variety of cultured cells, including human endothelial cells, fibroblasts, and sarcoma cells (Laiho et al., 1986; Rheinwald et al., 1987; Pöllanen et al., 1987; Levin and Santell, 1987), a finding that indicates the potential for efficient control of plasminogen activation in the growth substratum of these cells. Recently, it was shown that PAI-1 is associated with its binding protein vitronectin in plasma, in the subcellular matrix of endothelial cells (ECM), and in platelets (Wiman et al., 1988; Declerck et al., 1988; Mimuro and Loskutoff, 1989; Preissner et al., 1989; Salonen et al., 1989). At least for cultured vascular endothelial cells, vitronectin in the ECM is sequestered from the serum-containing growth medium and shown to be a requirement for PAI-1 deposition in the ECM (Seiffert et al., 1990; Preissner et al., 1990). In vivo, the presence of vitronectin in the vessel wall is undisputed, but its origin has not been established (Guettier et al., 1989; Niculescu et al., 1989). It should be noted that in vivo vitronectin may be sequestered from the serum as well or, alternatively, synthesized by cells constituting the vessel wall.

Recently, we reported that vitronectin endows PAI-1 with an additional specificity for thrombin and increases the second-order association rate constant with this protease by about two orders of magnitude (Ehrlich et al., 1990). In contrast, we confirmed that vitronectin does not alter the rate constant of PAI-1 with its target protease t-PA (Declerck et al., 1988). These findings together with the aforementioned considerations prompted us to study the interaction of thrombin with the authentic ECM of cultured endothelial cells. Consequently, we investigated whether PAI-1, deposited in the ECM and colocalized with vitronectin (Mimuro and Loskutoff, 1989), would be the "target" of thrombin. Here, we demonstrate that, upon administration of procoagulant thrombin to ECM, serpin-typical, SDS-resistant complexes are generated exclusively with PAI-1 and that vitronectin is required as a cofactor for this interaction. Procoagulant thrombin depletes PAI-1 from ECM via complex formation and proteolytic cleavage, thereby promoting the activity of plasminogen activators. In the fibrinolytic system, it has been recognized that thrombin indirectly enhances t-PA–induced plasminogen activation by the generation of fibrin, the obligatory cofactor for the enzyme (for review see Collen, 1980). In this report, we provide evidence for yet another pathway of thrombin-mediated promotion of plasminogen activator activity; i.e., by specific neutralization of ECM-associated PAI-1.

Materials and Methods

Materials

[^{35}S]Methionine (in vivo cell-labeling grade) and ^{125}I-Na were purchased from Amersham International PLC (Amersham, U. K.). The chromogenic substrates *H*-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide-dihydrochloride (S2288) and *H*-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide-dihydrochloride (S2238) were obtained from KabiAB (Stockholm, Sweden). Hirudin, BSA, diisopropyl fluorophosphate, the active site titrant *p*-nitrophenyl *p'*-guanidinobenzoate, and heparin from porcine intestinal mucosa were from Sigma Chemical Co. (St. Louis, MO). QAE-Sephadex and cyanogen bromide–activated Sepharose 4B was from Pharmacia Fine Chemicals (Uppsala, Sweden).

Purified Proteins

The concentration of purified human α-thrombin (4.5 mg/ml) was determined as described (Bradford, 1976). Active site titration of α-thrombin with *p*-nitrophenyl *p'*-guanidinobenzoate (Chase and Shaw, 1970) indicated a concentration of 5.0 mg/ml, a value which was used throughout the experiments. Radioiodination of thrombin (25 μg) with ^{125}I-Na (1 mCi) was performed using the Iodogen method and resulted in a specific activity of 30 μCi/μg protein. ^{125}I-labeled thrombin comigrated with unlabeled thrombin on SDS–polyacrylamide gels and retained (*a*) its catalytic activity towards S2238 and (*b*) its ability to rapidly form SDS-resistant complexes with antithrombin III in the presence of 0.5 U/ml heparin (Ehrlich et al., 1991). Two-chain Bowes melanoma t-PA (910,000 U/mg) was purchased from Biopool (Umea, Sweden). High molecular weight u-PA, purified from human urine, was from Calbiochem Corp., (La Jolla, CA). Thrombomodulin was purified to apparent homogeneity from rabbit lungs as described (Galvin et al., 1987). Briefly, the homogenates were extracted with Triton X-100 and adsorbed on QAE-Sephadex. The active fractions were pooled and immunopurified on immobilized antithrombomodulin antibody, followed by chromatography on DIP-thrombin-Sepharose and, finally, by Mono Q chromatography.

Antisera

The murine monoclonal antibodies CLB-1C3 and CLB-1B10, directed against human PAI-1 were a gift from Dr. J. A. van Mourik and Dr. H. Lambers (Department of Blood Coagulation, CLB Amsterdam). Additional monoclonal anti-human PAI-1 antibodies (MAI-12 and Sepharose-coupled MAI-13) were purchased from Biopool. Murine monoclonal antibody CLB-t-PA-6, directed against "kringle 1" of t-PA, was prepared and mapped as described (Van Zonneveld et al., 1986). Murine monoclonal antibodies aATIII-24, directed against human antithrombin III, and URO-4, directed against human u-PA, were the kind gift of Dr. Janny Abbink and Jan-Paul de Boer from the Department of Autoimmune Diseases (CLB, Amsterdam). Polyclonal antisera against purified human vitronectin were raised in rabbits (Ehrlich et al., 1990) and were fractionated by ammonium sulphate precipitation and ion-exchange chromatography on DEAE-Sephacel. Immunoglobulins from a control rabbit that had not been immunized were prepared in an identical fashion.

Rabbit immunoglobulins, raised against purified human von Willebrand factor, were from DAKOPATTS (Copenhagen, Denmark).

Preparation of Dishes Coated with ECM of Cultured HUVECs

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured in 75-cm^2 flasks as described (Jaffe et al., 1973; Willems et al., 1982). Second- or third-passage HUVECs were grown in 6-well plates or 25-cm^2 flasks until they reached confluency. 2–3 d later, the cells were removed as follows. The cells were washed three times with PBS (2 ml/well per wash or 10 ml/flask per wash) and then incubated for 10 min at 20°C with PBS containing 0.5% (vol/vol) Triton X-100 to remove the cells. The remaining ECM was washed three times with PBS and, additionally, twice with TST (20 mM Tris-HCl, pH 7.4, containing 100 mM NaCl and 0.1% [vol/vol] Tween-80). After this treatment, no visible cell debris was left in the wells as judged by light microscopy.

Complex Formation between ^{125}I-Thrombin and ECM Proteins

^{125}I-labeled human α-thrombin (final concentration: 0.3 nM) in TST was incubated with ECM at 20°C on a horizontal rotating platform (25 rpm). At designated times, ranging from 2.5 to 60 min, the supernatant was removed. Both the supernatant and (SDS-dissolved) ECM were separately subjected to 10% (wt/vol) SDS-PAGE. In some experiments, the ECM was

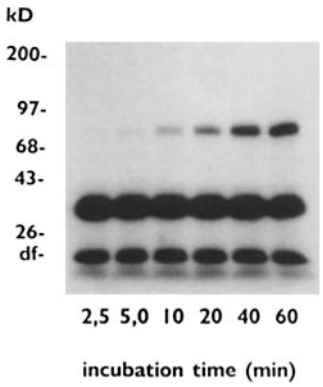

Figure 1. Time course of the appearance of SDS-stable complexes between ^{125}I-labeled thrombin and a protein from ECM. ^{125}I-labeled thrombin (0.3 nM) was incubated in TST buffer with ECM. At time points indicated, supernatant was removed from ECM and analyzed by SDS-PAGE and autoradiography.

preincubated for 15 min at 20°C with different antibodies on a horizontal rotating platform (25 rpm), before the addition of the radiolabeled thrombin.

Metabolic Labeling of ECM Proteins

HUVECs, grown to confluency in 25-cm^2 flasks, were incubated for 2 h at 37°C with 2 ml of glutamine- and methionine-deficient RPMI 1640, supplemented with transferrin (0.02 mg/ml), insulin (0.01 mg/ml), human serum albumin (5.0 mg/ml), glutamine (0.3 mg/ml), and [^{35}S]methionine (10 μCi/ml; specific activity >1,000 Ci/mmol). Then, unlabeled L-methionine was added (final concentration: 50 mM) for a chase period of 30 min, after which the cells were removed as described above. PAI-1 represented the predominant radiolabeled protein band in the matrix (data not shown).

Interaction of t-PA, u-PA, and Thrombin with Metabolically Labeled PAI-1 from ECM

After detergent-induced removal of HUVECs, the proteases were added to the supernatant (TST buffer) of metabolically labeled ECM. Incubation was carried out at 20°C for various times and terminated by removal of the supernatants from the culture dishes. Aliquots of these supernatants were then immunoprecipitated with the appropriate antibodies coupled to Sepharose, and the immunoprecipitable fractions were analyzed by SDS-PAGE and fluorography as described below.

SDS-PAGE

Electrophoretic separation of polypeptides was performed using 10% polyacrylamide gels as described (Laemmli, 1970). Samples were analyzed under nonreducing conditions in 0.05 M Tris-HCl, pH 6.8, 2% (wt/vol) SDS, 8% (vol/vol) glycerol, and 0.01% (wt/vol) Bromophenol Blue, except when indicated otherwise. The relative migration of the radiolabel was visualized by autoradiography (for ^{125}I) or fluorography (for ^{35}S) and compared with the migration of prestained molecular weight markers (Gibco Laboratories, Grand Island, NY; Bethesda Research Laboratories, Gaithersburg, MD).

Immunoprecipitation

Murine monoclonal antibodies, used for immunoprecipitations as indicated in the text, were coupled to cyanogen bromide–activated Sepharose 4B according to the instructions provided by the manufacturer (10 mg protein/g Sepharose). The Sepharose beads were blocked by incubation for 2 h at 4°C with 0.2 M glycine, pH 8.0, and stored at 4°C in PBS containing 0.04% (wt/vol) sodium azide. Immobilized antibodies (25 μl of packed beads) were incubated 12–16 h with 75 μl of the respective sample. The beads were washed three times with 1 ml TST buffer, followed by two washes with 1 ml TST containing 500 mM NaCl and, finally, with 1 ml TST buffer. Immunoprecipitated proteins were solubilized in 50 μl gel sample buffer and 25 μl was subjected to SDS-PAGE and, subsequently, to autoradiography or fluorography.

Influence of ECM on Amidolytic t-PA Activity

HUVECS (third passage) were plated into 96-well plates and grown to confluency. Cells were removed with 0.5% (vol/vol) Triton X-100 as described above, and 50 μl TST buffer containing 10 mg/ml BSA was added to ECM in each well. The wells then received either no compound or the inhibitory monoclonal anti-PAI-1 antibody (MAI-12, 80 μg/ml) or thrombin (30 or 300 nM) and incubation was allowed to proceed for 30 min. The supernatants were taken off; the wells were washed three times with 1 ml TST buffer containing 10 mg/ml BSA; and 50 μl of this buffer containing 40 U/ml hirudin (to quench residual thrombin activity) was added to each well. Then, 5 μl of t-PA (final concentration: 3 nM) was added into the wells containing the pretreated matrices. After a 30-min incubation, 25 μl of the supernatants was added to 200 μl 0.5 mM S2288 and the optical density at 405 nm was recorded every 3 min using a Titertek Twinreader (Flow Laboratories, Irvine, U.K.). The values of control incubations, without added t-PA, were subtracted from the values of the respective samples.

Results

Complex Formation between a Protein from ECM and Thrombin

To study whether thrombin would form SDS-stable complexes with any compound present in the ECM, ^{125}I-labeled thrombin was added to ECM which remained attached to the bottom of tissue culture flasks after removal of the endothelial cells. After an incubation, ranging from 2.5 to 60 min, the supernatants as well as SDS-detached ECMs were analyzed separately by SDS-PAGE and autoradiography. Fig. 1 shows the time-dependent appearance in the supernatant of ECM of SDS-stable complexes between thrombin and another protein. A faint complex band is visible after 2.5 min of incubation which, upon longer incubation, increases in intensity and reaches optimal complex formation after about 60 min. Free as well as complexed thrombin are almost exclusively (>95%) recovered in the supernatant of ECM (data not shown), indicating that (*a*) the large majority of the thrombin does not bind to ECM and (*b*) after formation of the SDS-stable complex between thrombin and a compound from the ECM, the complex dissociates from the ECM. The apparent molecular weight of the complex is ~80,000. This value corresponds with a 1:1 equimolar complex between thrombin and an ECM protein with an apparent molecular weight of ~44,000. Clearly, the apparent molecu-

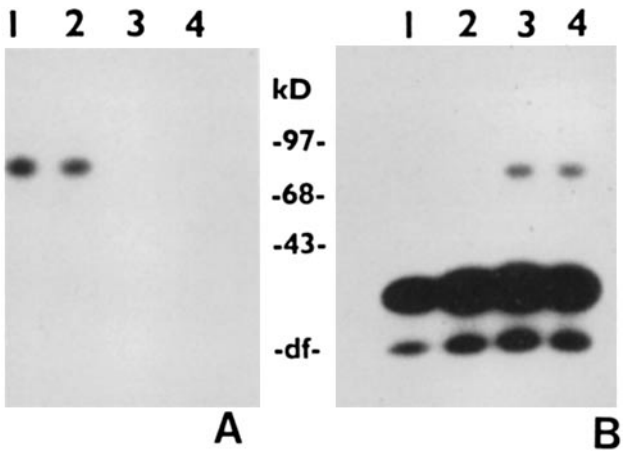

Figure 2. Immunological identification of the ECM protein forming SDS-stable complexes with ^{125}I-labeled thrombin. Aliquots of the supernatant of a 60 min incubation of ^{125}I-labeled thrombin with ECM were immunoprecipitated with the following murine monoclonal antibodies: (lane *1*) MAI-13 (anti-PAI-1); (lane *2*) CLB-1C3 (anti-PAI-1); (lane *3*) CLB-t-PA 6 (anti-t-PA); and (lane *4*) aATIII-24 (anti-antithrombin III). Immunoprecipitable (*A*) and non-precipitable (*B*) fractions were analyzed by SDS-PAGE and autoradiography.

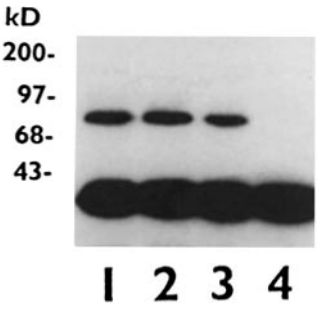

Figure 3. Effect of monoclonal PAI-1 antibodies on the formation of SDS-stable complexes between ^{125}I-labeled thrombin and ECM-associated PAI-1. ECM was incubated with antibodies (6 μg/ml) against PAI-1. After 15 min, ^{125}I-labeled thrombin (0.3 mM) was added, and the incubation was allowed to proceed for 60 min. The supernatants were then analyzed by SDS-PAGE and autoradiography. (Lane *1*) No antibody; (lane *2*) CLB-1C3; (lane *3*) CLB-1B10; (lane *4*) MAI-12. Three independent experiments gave similar results.

lar weight of PAI-1, being a predominant protein in the ECM, would fit as the counterpart of thrombin in the observed complex.

Identification of the ECM Component

To assess whether the ECM compound forming serpin-typical SDS-stable complexes with thrombin is identical to PAI-1, part of the supernatant of a 60-min incubation of ^{125}I-labeled thrombin with ECM was immunoprecipitated with two different monoclonal antibodies against PAI-1 and, as a control, with monoclonal antibodies against antithrombin III and t-PA. The results are depicted in Fig. 2. The SDS-stable complexes can be quantitatively immunoprecipitated with either one of the two monoclonal antibodies directed against PAI-1 (lanes *1* and *2*), whereas the control antibodies fail to precipitate the complexes (lanes *3* and *4*). As expected, uncomplexed thrombin is not precipitated with any of the antibodies. Consequently, thrombin forms complexes exclusively with PAI-1 present in the ECM.

Effect of Anti-PAI-1 Monoclonal Antibodies on Complex Formation

Inhibition of the target enzymes t-PA and u-PA by PAI-1 requires the functionality of the carboxyl-terminally located reactive center of the inhibitor. To establish whether the same structural motif is implicated in the formation of SDS-stable complexes with thrombin, the experiments were carried out in the presence of a monoclonal antibody (MAI-12) that inhibits the interaction between PAI-1 and plasminogen activators (Keijer et al., 1991). Noninhibitory anti-PAI-1 antibodies served as control. The results are shown in Fig. 3. Formation of PAI-1–thrombin complexes is observed in the absence of antibody (lane *1*). A preincubation of ECM for 15 min at 20°C with the noninhibitory antibodies CLB-1C3 and CLB-1B10 results in a similar extent of complex formation (lanes *2* and *3*, respectively). In contrast, preincubation of ECM with the monoclonal anti-PAI-1 antibody MAI-12 completely abolishes the formation of SDS-stable complexes between thrombin and ECM-associated PAI-1, demonstrating the requirement of a functionally reactive center for complex formation with thrombin.

Effect of Antivitronectin Antisera on Complex Formation

In a purified system, the rate of interaction between PAI-1 and thrombin is greatly accelerated by the cofactor activity of vitronectin (Ehrlich et al., 1990). Therefore, to further characterize the interaction between thrombin and PAI-1 in the growth substratum of endothelial cells, the effect of a polyclonal antiserum, raised against purified human vitronectin, on the complex formation between thrombin and PAI-1 was investigated. ^{125}I-labeled thrombin was incubated for 60 min with ECM that had been pretreated with antivitronectin antiserum or with control antisera. As shown in Fig. 4, preincubation of ECM with antivitronectin antiserum (lane *2*) resulted in a substantial decrease of complex formation as compared with the controls; i.e., preincubation of ECM in the absence of antisera (lane *1*), a polyclonal antiserum against human von Willebrand factor (lane *3*), or preimmune serum from the rabbit used to generate the antivitronectin antiserum (lane *4*). Thus, ECM vitronectin performs an essential role to sustain complex formation between PAI-1 and thrombin.

Effect of Different Proteases on ECM-associated ^{35}S-Labeled PAI-1

Next, we studied the effect of thrombin on PAI-1, de novo synthesized and deposited into the growth substratum, and compared it with the effect of t-PA and u-PA. To that end, the endothelial cells were metabolically labeled for 2 h with [^{35}S]methionine, followed by a chase period of 30 min. Cells were then removed as described and the resulting growth substrata were incubated for 1 h with increasing amounts of either one of the unlabeled proteases thrombin, t-PA or u-PA. During a 1-h incubation in the absence of proteases we observed a minor release of radiolabeled proteins into the supernatant (data not shown). As depicted in Fig. 5 one of these proteins, displaying an apparent molecular weight of 44,000, is specifically immunoprecipitated with the anti–PAI-1 monoclonal antibody MAI-13, indicating that this protein is immunologically related to PAI-1. In the presence of either t-PA, u-PA, or thrombin, the dose-dependent appearance of three major immunoprecipitable polypeptides can be noted. The two lower bands migrate at an identical position with apparent molecular weights of 44,000 and 40,000, irrespective of the protease used. In contrast, the ap-

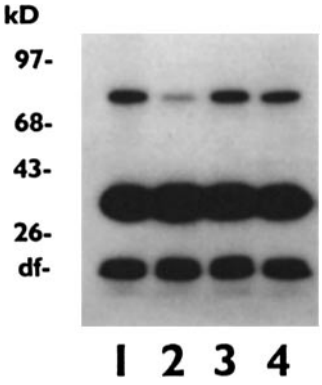

Figure 4. Effect of antivitronectin antiserum on the formation of SDS-stable complexes between ^{125}I-labeled thrombin and PAI-1. ECM was preincubated with different antisera (total protein: 22.5 μg/500 μl per well of a 6-well plate) for 15 min, at which time ^{125}I-labeled thrombin was added, and the incubation was allowed to proceed for another 60 min. The supernatants of the respective incubations were applied to SDS-PAGE and autoradiography. (Lane *1*) No immunoglobulins; (lane *2*) immunoglobulins from a rabbit immunized with human vitronectin antiserum; (lane *3*) immunoglobulins from a rabbit immunized with human von Willebrand factor antiserum; and (lane *4*) immunoglobulins from a control (not immunized) rabbit. Three independent experiments gave similar results.

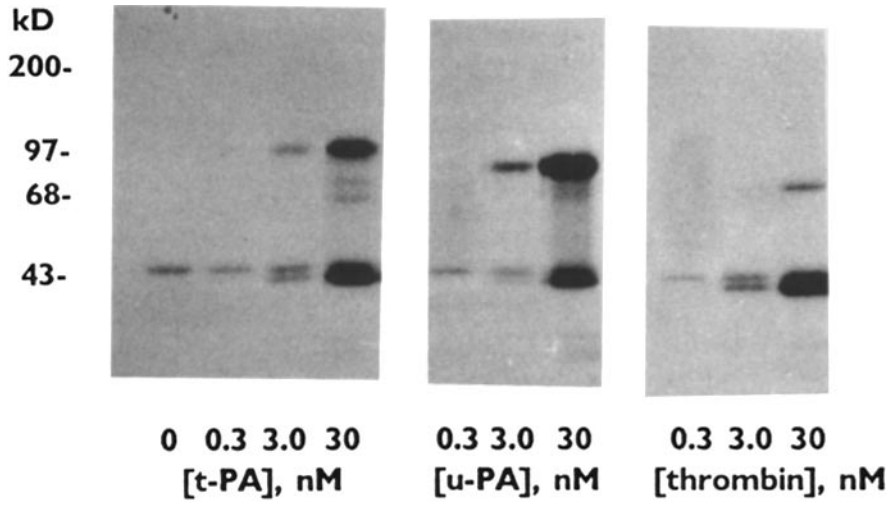

Figure 5. Effect of proteases on ECM-associated, ^{35}S-labeled PAI-1. ECM, metabolically labeled with [^{35}S]methionine, was incubated for 60 min with different concentrations t-PA, u-PA, or thrombin as indicated. Aliquots of the respective supernatant were immunoprecipitated with monoclonal antibody MAI-13 (anti-PAI-1), and immunoprecipitable fractions were subjected to SDS-PAGE and fluorography.

parent molecular weight of the largest of the three bands was strictly dependent on the protease used, being approximately 110,000 in the case of t-PA, 95,000 with u-PA, and 80,000 with thrombin. These findings, together with the observation that all three bands could be immunoprecipitated with the anti-PAI-1 monoclonal antibody MAI-13, are consistent with the notion that the indicated three bands represent three different forms of PAI-1; i.e., (from bottom to top) cleaved inactive PAI-1, native PAI-1, and a complex of PAI-1 and the respective protease. However, a striking difference can be observed between the proteases with respect to the relative quantities of complexed and cleaved PAI-1. With u-PA, the majority of PAI-1 eluted from ECM is in a serpin-typical, SDS-resistant complex. However, with t-PA the amount of cleaved PAI-1 approximates the amount of PAI-1 being in an SDS-stable protease–protease inhibitor complex, whereas with thrombin, the majority of the PAI-1 eluted from ECM is present in the cleaved, inactive form. It should be kept in mind, that (part of) the 40,000-mol wt cleaved form of PAI-1 may be generated from t-PA–PAI-1 or thrombin–PAI-1 complexes by boiling the immunoprecipitates in SDS buffer before electrophoresis, an issue that will be dealt with in a following paragraph.

Requirement for the Procoagulant Activity of Thrombin to Deplete ECM-associated PAI-1

Since α-thrombin can display either a procoagulant or an anticoagulant activity, we determined the required properties of the thrombin preparation to form complexes with PAI-1. For that purpose, thrombin was preincubated either with diisopropyl fluorophosphate, hirudin, or thrombomodulin. After diisopropyl fluorophosphate treatment, <0.1% of the thrombin remained amidolytically active towards the chromogenic substrate S2238, whereas preincubation with hirudin resulted in a residual amidolytic activity of <1% (data not shown). Thrombin in complex with rabbit thrombomodulin fully retained its amidolytic activity, however, the

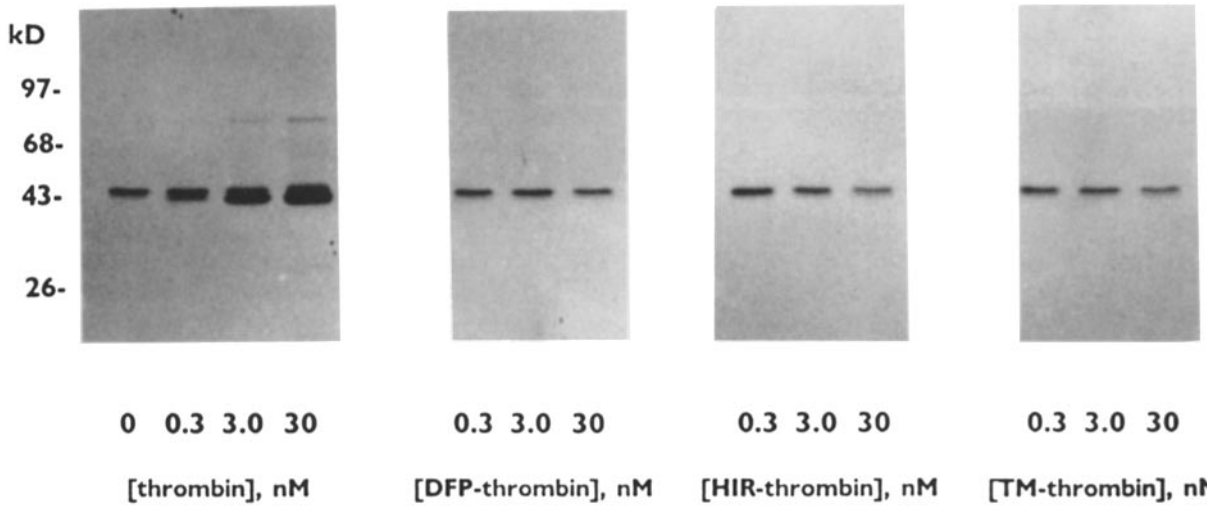

Figure 6. Effect modulation of thrombin activity on the interaction with ECM-associated ^{35}S-labeled PAI-1. Metabolically labeled ECM was incubated for 30 min with different concentrations of thrombin; DFP-treated thrombin (*DFP-thrombin*), hirudin-treated thrombin (*HIR-thrombin*), or thrombin in complex with its endothelial cell surface receptor thrombomodulin (*TM-thrombin*) as indicated. Immunoprecipitable (monoclonal anti-PAI-1 antibody MAI-13) fractions of the respective supernatants were subjected to SDS-PAGE and fluorography.

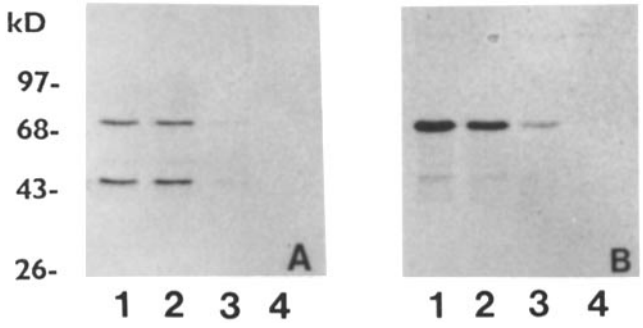

Figure 7. Functional neutralization of PAI-1 activity in ECM by procoagulant thrombin. Metabolically labeled ECM was incubated for 60 min with the following concentrations of unlabeled thrombin: 0 nM (lanes *1*); 3 nM (lanes *2*); 30 nM (lanes *3*); 300 nM (lanes *4*). The supernatants were removed and the matrices were washed twice with TST buffer. Then, t-PA (*A*) or u-PA (*B*) to a concentration of 30 nM were added and incubated with the pretreated ECMs for an additional 60 min, after which time the supernatants were removed, immunoprecipitated with monoclonal anti-PAI-1 antibody MAI-13, and subjected under reducing conditions to SDS-PAGE and fluorography.

macromolecular specificity was shifted from procoagulant to anticoagulant, as exemplified by efficient activation of human protein C (data not shown). The results of complex formation of these four different thrombin preparations with ^{35}S-labeled PAI-1, de novo synthesized and deposited in ECM, are shown in Fig. 6. Consistent with the results presented in Fig. 5, procoagulant thrombin effectively eluted PAI-1 from ECM, with a minor portion of the immunoprecipitable PAI-1 being in an SDS-resistant complex with the protease and the major fraction corresponding with the apparent molecular weight of 40,000 of the cleaved form. In contrast, none of the modified forms of thrombin eluted PAI-1 from ECM by complex formation and/or cleavage, indicating that the procoagulant protease moiety of thrombin is strictly required for specific complex formation and, consequently, neutralization of PAI-1.

Promotion of Plasminogen Activator Activity in ECM by Procoagulant Thrombin

The effect of the interaction between thrombin and matrix-associated PAI-1 on subsequent control of plasminogen activation in ECM was studied in two systems. In the first experiment, metabolically labeled ECM was preincubated with increasing concentrations of thrombin, followed by incubation with t-PA or u-PA and immunoprecipitation of the respective supernatants, using antiprotease antibodies. The results are shown in Fig. 7. If thrombin was absent during the preincubation, both t-PA and u-PA eluted metabolically labeled PAI-1 from ECM by complex formation. In accord with our previous observations, these complexes were almost completely SDS stable in the case of u-PA, whereas with t-PA, approximately half of the radiolabeled PAI-1 dissociates from the complex during preparation of the sample in gel loading buffer. However, after preincubating the matrices with increasing amounts of thrombin, the number of PAI-1–t-PA or PAI-1–u-PA complexes formed during subsequent incubation of ECM with these plasminogen activators decreases in a dose-dependent manner.

A different experimental approach was used to show that preincubation of ECM with thrombin neutralizes PAI-1 activity and thereby abrogates the capacity of ECM to inhibit t-PA. To that end, ECM was preincubated with different compounds as outlined below and the subsequent effect of these pretreated ECM's on the activity of t-PA toward the synthetic substrate S2288 was investigated. The results are shown in Fig. 8. Incubation of t-PA with untreated ECM (*A*) resulted in a 45% inhibition of the amidolytic activity as compared with the control; i.e., incubation of t-PA with ECM with the monoclonal anti-PAI-1 antibody MAI12 (*B*), which inhibits the activity of PAI-1. A similar effect as the one observed with the antibody, i.e., prevention of the inhibitory capacity of PAI-1, was seen upon preincubating ECM with thrombin. A concentration of 30 nM resulted in partial prevention of the inhibition executed by PAI-1 (*C*), whereas with 300 nM of thrombin complete neutralization of PAI-1 was observed (*D*).

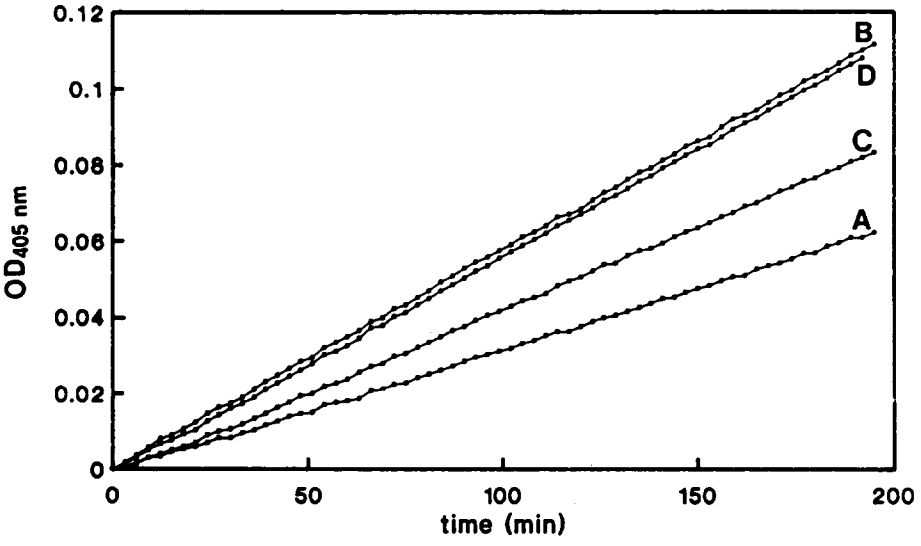

Figure 8. Effect of thrombin on inhibition of t-PA activity by ECM. ECM in 96-well plates, pretreated differently as indicated below, was incubated for 30 min with t-PA (3 nM). Residual t-PA activity in the wells was determined by adding aliquots to the chromogenic substrate S2288 and recording the increase of optical density at 405 nm. Preincubation was performed for 30 min with buffer alone (*A*), inhibitory anti-PAI-1 monoclonal antibody MAI-12 (*B*), 30 nM thrombin (*C*), or 300 nM thrombin (*D*). Experiments were run in triplicate with standard deviations of <5%.

Discussion

In this report, we studied the functional interaction between ECM-associated PAI-1 and exogenously added thrombin. For this purpose, ECM formed by cultured human endothelial cells was used. In this ECM, largely resembling the basement membrane of the vessel wall with respect to composition and structure (Gospodarowicz et al., 1980; Vlodavsky et al., 1980), PAI-1 is one of the abundant proteins, emphasizing the potential of this compartment for regulation of events associated with protease activity. Administration of ^{125}I-labeled procoagulant thrombin to ECM results in SDS-resistant complexes between the protease and PAI-1 present in ECM, followed by a rapid release of these complexes into the supernatant. These observations are reminiscent of the behavior of t-PA/PAI-1 complexes described in previous studies (Barnathan et al., 1988; Schleef et al., 1990). Furthermore, we demonstrate that depletion of PAI-1 from the ECM by thrombin causes subsequent promotion of the activity of a plasminogen activator, which may ultimately lead to increased plasmin generation.

Formation of serpin-typical complexes between PAI-1 and procoagulant thrombin in the growth substratum of cultured endothelial cells has not been described previously. In an experimental approach similar to the one used here, Knudsen et al. (1987) incubated ^{125}I-labeled thrombin with the matrix of bovine smooth muscle cells. Although PAI-1 is also a predominant protein in the substratum of these cells, the investigators did not observe SDS-resistant complexes between PAI-1 and thrombin. The apparent discrepancy with our findings may be related to differences in the composition of the matrices generated by these different cell types. Indeed, as initially observed with purified components, the efficient formation of thrombin–PAI-1 complexes is dependent on the presence of cofactors such as vitronectin or heparin (Ehrlich et al., 1990, 1991). In agreement with these findings, it was demonstrated that PAI-1 in the ECM is associated with its binding protein vitronectin (Preissner et al., 1990; Seiffert et al., 1990) and that these complexes appear to be essential also for maintenance of the adherent cell phenotype as recently demonstrated for the human fibrosarcoma cell line HT-1080 (Ciambrone and McKeown-Longo, 1990). The distribution and concentration of PAI-1–vitronectin complexes may differ between substrata generated from different cell types and may explain differences in association between thrombin and PAI-1.

A prerequisite for a functional interaction in vivo between thrombin and PAI-1–vitronectin complexes in the subendothelial structures is the accessibility of these complexes for the protease. Endothelial cells are able to assemble the proteases and cofactors of the coagulation system on their surface, ultimately resulting in the generation of thrombin (Stern et al., 1985). Thrombin can penetrate into subendothelial layers not only when subendothelium is exposed to the circulation, but also when the integrity of the endothelial cell layer is maintained (Garcia et al., 1986). In view of the morphological alterations of the endothelial cell monolayer, observed upon administration of thrombin (Laposata et al., 1983), it is conceivable that the subendothelial matrix is a particularly susceptible target for thrombin. Having penetrated the endothelial cell monolayer, thrombin participates in several physiological processes other than coagulation, notably proliferation and chemotaxis of inflammatory cells as well as proteolytic degradation of basement membrane proteins, leading to cell invasion and metastasis (Chen and Buchanan, 1975; Bar-Shavit et al., 1983*a*,*b*; Bar-Shavit and Wilner, 1986). Its critical involvement in such a variety of physiological processes, together with the potentially devastating effects on the organism resulting from excessive proteolysis, requires rigorous regulation of the activity of thrombin. In vivo, the procoagulant activity of thrombin is controlled by two strikingly different mechanisms: (*a*) modulation of the macromolecular specificity of thrombin by its endothelial cell surface receptor thrombomodulin, turning thrombin into an anticoagulant protein (Esmon, 1989) and (*b*) inhibition of its activity by specific serpins (antithrombin III [Rosenberg and Damus, 1973]), heparin cofactor II (Tollefsen and Blank, 1981), and protease nexin I (Baker et al., 1980). The reaction with these serpins is dramatically accelerated by heparin. However, these regulatory factors and cofactor may not be available or not operative at certain locations where thrombin can be found. For example, the endothelial cells used in this study had been cultured in the presence of serum, containing for example antithrombin III; and anticoagulantly active heparin-like glycosaminoglycans are synthesized by endothelial cells (Marcum and Rosenberg, 1985). However, no complexes between thrombin and antithrombin III–heparin were encountered in the present study. This indicates that this serpin had not been sequestered from the medium and/or heparin-like molecules are not deposited in ECM. Rather, in this environment PAI-1–vitronectin complexes may well serve as a unique regulatory system for thrombin activity.

In addition to a role of PAI-1–vitronectin complexes in the regulation of the degradation of basal membrane components by serine proteases, it can be envisaged that such complexes may provide a new link between coagulation and the fibrinolytic system. Both systems are intimately connected at the level of thrombin activity as evidenced by the present as well as by several other observations. The influence of the fibrinolytic system on coagulation is substantiated by the finding that during thrombolytic therapy with t-PA, there is a transient increase in thrombin in plasma (Owen et al., 1988). This increase was speculated to be due to either the initiation of the prothrombinase complex or to the release of preexisting thrombin from the dissolving thrombus. The latter hypothesis might explain the high incidence of reocclusion after successful coronary thrombolysis, considering the high concentration of thrombin in a thrombus (up to 140 nM; Walz et al., 1985) and the resistance of such fibrin-bound thrombin to inhibition by antithrombin III–heparin (Hogg and Jackson, 1989; Weitz et al., 1990). Visa versa, thrombin has the potential to affect the fibrinolytic system. Here, however, the situation appears to be more complex, since the effect of thrombin on fibrinolysis may be in opposite directions; i.e., inhibition as well as stimulation. Inhibition by thrombin may be related to (*a*) activation of platelets, resulting in the release of active PAI-1 (Erickson et al., 1984) and of PAI-1–vitronectin complexes (Preissner et al., 1989), and (*b*) increased release of PAI-1 from HUVECs (Gelehrter and Szyncer-Laszuk, 1986; Grulich-Henn and Müller-Berghaus, 1990). In vivo, elevated levels of active PAI-1 may complex with the molar excess of plasma vitronectin and may, presumably locally, affect the balance between t-PA ac-

tivity and that of procoagulant thrombin. Conversely, thrombin promotes fibrinolysis in several ways, e.g., by (*a*) indirect stimulation of t-PA–mediated plasminogen activation by the generation of fibrin, the obligatory cofactor of t-PA (for review see Collen, 1980) and (*b*) stimulation of t-PA synthesis in and release from (cultured) endothelial cells (Levin et al., 1984). A similar observation has been made in vivo, using experimental animals (Giles et al., 1989). Intravenous administration of mixtures, consisting of activated factor X and phosphatidylcholine-phosphatidylserine, obviously causing the generation of thrombin, resulted in a rapid, almost 1,000-fold increase in plasma levels of t-PA. In conclusion, there is a variety of (partly opposite) effects that interconnect the fibrinolytic system and coagulation. Specifically, the data presented here are indicative of a direct interaction between thrombin, the key enzyme in coagulation, and PAI-1, the major protein controlling the activity of t-PA and u-PA. The addition of procoagulant thrombin to ECM, containing a high local concentration of PAI-1–vitronectin complexes, results in a mutual neutralization of both PAI-1 and thrombin. The interaction between ECM-associated PAI-1–vitronectin complexes and procoagulant thrombin constitutes an important regulatory function for all subsequent processes that involve thrombin-promoted, as well as plasminogen activator–promoted, modification of the subendothelial matrix.

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