

Monocyte Plasminogen Activator Inhibitor 2 (PAI-2) Inhibits u-PA-mediated Fibrin Clot Lysis and Is Cross-linked to Fibrin

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Summary

Plasminogen activator inhibitor 2 (PAI-2) is a major product of activated human monocytes. Here we show that monocytes inhibited u-PA- but not t-PA-mediated fibrinolysis, by secreting PAI-2 into an overlying fibrin clot. Extracts of arterial and venous human thrombi were found to contain active PAI-2. PAI-2 was cross-linked to fibrin in a reaction catalyzed by two major transglutaminases (TG), tissue TG and factor XIII. The activity of PAI-2 was not affected by such cross-linking. Cross-linking of PAI-2 to fibrin was inhibited by Tridegin, a specific inhibitor of TG, and also by EDTA and iodoacetamide. The use of competitive peptides mimicking the loop between helices C and D of PAI-2 identified Gln 83 and 86 as residues important in cross-linking. This study defines a mechanism by which PAI-2 is localized to fibrin, where it acts as an effective inhibitor of u-PA-mediated fibrinolysis.

Introduction

Fibrin is deposited as a result of the coagulation cascade, and persistence of fibrin is dependent on the local balance of plasminogen activators (PAs) and plasminogen activator inhibitors (PAIs). Plasmin, the active serine protease that mediates degradation of fibrin, is generated from the zymogen, plasminogen, by urokinase (u-PA) or tissue-type PA (t-PA). PAI-1 and PAI-2 inhibit plasmin generation by formation of a 1:1 stoichiometric complex with u-PA and t-PA (reviewed in 1). Persistence of fibrin in local environments such as the atherosclerotic plaque indicates an imbalance in fibrinolysis (2). Increased PAI-1 is one such imbalance, and it has been shown that PAI-1 mRNA and antigen are increased in the atherosclerotic vessel wall and also in human thrombi (3-6). PAI-2 has recently been found to be up-regulated in the atherosclerotic plaque (7). PAI-2, unlike PAI-1, is not detected in normal plasma but is detected during pregnancy (8). The distribution of PAI-2 *in vivo* has not been fully established. However, it is likely that local production of PAI-2 in various tissues is important, and PAI-2 has been detected in the epidermis, in inflamed tissues and in the placenta (9-11). The cell types that express PAI-2 in these situations are keratinocytes, monocytes, and trophoblasts, respectively. There is much

more information available on the expression and regulation of PAI-2 *in vitro*. Many cell types express PAI-2, including monocytes, endothelial cells, smooth muscle cells, fibroblasts and many transformed cell lines. The gene for PAI-2 is modulated by many different stimuli (reviewed in 12). PAI-2 is a member of a sub-family of serpins, the ov-serpins, named for their archetypal member, ovalbumin (13). This family is characterized by a number of features, including the lack of a cleavable sequence to direct secretion through the ER-Golgi system; many of these proteins are therefore present predominantly as intracellular molecules. The location of PAI-2 appears to be cell-type specific, and endothelial cells and fibroblasts maintain PAI-2 almost exclusively as an intracellular molecule, whereas monocytes have both intracellular and secreted PAI-2 (14-16). It is notable that when secretion does occur from monocytes, it does so by an ER-Golgi independent system, reminiscent of but not identical to that for interleukin 1 β (17). Dual location of PAI-2 may reflect different functions in the two sites. PAI-2 secreted by the monocyte efficiently modulates u-PA-mediated migration of cells (18), whereas intracellular PAI-2 has been shown to prevent cells from undergoing apoptosis; the mechanism for this inhibition is unknown (19-21). PAI-2 has a unique extended loop of 33 amino acids between helices C and D. This loop is distant from the active site, and contains three glutamine residues that act as substrates for transglutaminases (22). This loop has been shown to be important for cross-linking of PAI-2 to trophoblast membranes and to Matrigel, a source of extracellular matrix (23). It has also been demonstrated that this interhelical loop is necessary for PAI-2 to inhibit apoptosis (21). PAI-2 has been shown to be cross-linked to structures within the cornified envelope of the epidermis, a thick deposit of protein that provides stability (24). Cross-linking is mediated by transglutaminases (TG), a ubiquitous family of enzymes that catalyze formation of a covalent amide bond between carboxamide groups of donor glutamine residues and acyl acceptors of primary amines. The amino group of lysine residues serves as a common acyl acceptor (25). Specificity of cross-linking is determined by residues surrounding the glutamine, and also by the particular TG enzyme (26). The members of the TG family include factor XIII (FXIII) and tissue transglutaminase (TTG). FXIII in plasma, monocytes and platelets catalyses cross-linking of the fibrin clot, resulting in formation of a stable insoluble clot (25). FXIII inhibits fibrinolysis by cross-linking α_2 -antiplasmin (α_2 -AP) to fibrin. This reaction inhibits plasmin-mediated degradation of the fibrin clot (27). Tissue TG has a broad tissue distribution and is found in extracellular matrices (25), where TTG may play a role in ECM/fibrin stabilization (28). The other important situation where TTG plays a role is apoptosis, where increased TG activity delays the process of programmed cell death (25). In this investigation, we have demonstrated that activated peripheral blood monocytes secreted PAI-2, which was incorporated into an overlying fibrin clot. PAI-2 efficiently inhibited u-PA-mediated fibrin clot lysis. PAI-2 was cross-linked to fibrin, in a

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transglutaminase-catalysed reaction that was dependent on glutamines 83 and 86. PAI-2 is an important inhibitor of u-PA-mediated fibrin lysis, and cross-linking of PAI-2 to fibrin may serve to localize its activity.

Methods

Isolation and culture of monocytes. Monocytes were isolated from human peripheral blood as previously described (16). Briefly, blood was collected, with informed consent, into 10% v/v of 3.8% w/v tri-sodium citrate. Blood was diluted with RPMI (Rosewell Park Memorial Institute) medium (Gibco BRL, UK) and layered onto Ficoll-paque (Pharmacia, UK). Blood samples were centrifuged, white blood cells were collected, and cells were washed three times with RPMI medium. The final cell pellet was resuspended in RPMI containing 10% v/v fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (RF10). Monocytes (5×10^4 monocytes/well) were isolated by preferential adherence to 96-well plates (Nunc, UK) by incubation at 37° C for 1 h in a 5% CO₂ environment. Non-adherent cells were removed, monocytes were washed three times with RPMI, and monocytes were incubated for 18 h with 200 µl per well of either RF10 or RF10 containing 10 ng/ml lipopolysaccharide (LPS). Endotoxin-free conditions were maintained throughout by the use of pyrogen-free disposable plasticware, solutions were sterilized through a 0.2 µm filter, and endotoxin was monitored by the *Limulus* amoebocyte lysate assay (Sigma). The monocyte-rich population contained 90 to 95% monocytes, as monitored by the non-specific esterase stain.

Lactate dehydrogenase (LDH) was assayed as a measure of cell death using the CytoTox⁹⁶ kit (Promega, UK), as previously described (16). Viability of cells was also studied using the Trypan blue membrane exclusion test.

Clot lysis assay on isolated monocytes. A microtitre plate fibrin clot lysis assay was performed on isolated monocytes (29). Monocytes were either untreated or stimulated with 10 ng/ml LPS for 18 h, the conditioned medium (200 µl) was removed, and stored for analysis at -70° C. Monocytes were washed twice with 171 mM NaCl, 3.4 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄ (PBS). Thrombin (0.4 U/ml) and calcium (5.3 mM) were added to wells, and this was followed by fibrinogen (2.94 µM, Kabi, Sweden) containing 0.24 µM plasminogen in 10 mM Tris, 0.88 mM KCl and 42.8 mM NaCl, pH 7.4, which resulted in a final volume of 100 µl/well. Single chain t-PA (20 pM, Genentech, USA), two-chain t-PA from melanoma cells (20 pM, TNO Gaubius Institute, Netherlands) or u-PA (180 pM, NIBSC, UK) was added to the fibrinogen/plasminogen mixture. The microtitre plate was incubated at 37° C for 30 min to allow clot formation. Lysis of the fibrin clot was monitored by the decrease in turbidity (405 nm) and expressed as the time taken to achieve 50% clot lysis. The involvement of monocyte PAI-2 or PAI-1 was studied by incorporation of IgG preparations of rabbit antibodies raised against PAI-2 (16), PAI-1 (30) or IgG from non-immune rabbit serum into the fibrin clot at a final concentration of 250 µg/ml. The solubilized clots were collected at 8 h and stored at -70° C for analysis, as were the monocytes, which were scraped from the microtitre plate into PBS. Experiments were performed on six occasions, on monocytes isolated from six individual donors, since donor variability between monocytes is a well-known phenomenon (16). Each condition was carried out in triplicate and data are shown from one experiment on a representative donor. Clot lysis in the absence of cells was assayed exactly as above. Recombinant PAI-1 (31) and rPAI-2 (32) (Delta Biotechnology, UK) were added as appropriate.

Preparation of human thrombi extracts. Extracts were prepared as previously described (6). Briefly, human thrombi were washed three times with 0.9% w/v NaCl, dried, fixed in 10% neutral buffered formalin and sectioned into 5 µm pieces using a cryostat. Frozen samples were cut into small pieces and ground into a fine powder, using a Mikro-Dismembrator (B Braun Biotech, Melsungen, Germany) and liquid nitrogen. The sample was extracted in 50 mM phosphate pH 7.0, 0.2 M epsilon amino-caproic acid, 1 M NaCl, 0.01% Tween 20, and was centrifuged at 11,600 g for 10 min. The supernatant was removed and the pellet was re-extracted. A total of three extractions were assayed for PAI-2. PAI-2 antigen (ng/g tissue) was expressed as a sum of these three extractions. All samples were stored at -70° C.

Measurement of PAI-2. PAI-2 antigen was assayed in conditioned medium, clots, and monocytes by enzyme-linked immunosorbent assay (ELISA), as previously described (16). Briefly, microtitre plates (Costar, UK) were coated with a monoclonal antibody to PAI-2 (MAI-21, Biostat, UK; 2 µg/ml). Recombinant PAI-2 (Delta Biotechnology) was diluted to give a standard curve of 0-10 ng/ml. PAI-2 was detected using an IgG preparation of a rabbit antibody to rPAI-2, and an antibody to rabbit IgG conjugated to alkaline phosphatase. Colour was generated by p-nitrophenol phosphate (2 mg/ml) in 0.1 M glycine buffer, pH 10.4, and absorbance was monitored at 405 nm.

The activity of rPAI-2 and rPAI-1 was measured by inhibition of u-PA-mediated cleavage of a chromogenic substrate, S-2444 (Chromogenix, UK) as described; the unit of inhibitor in each case was defined as that which inhibits 1 IU of u-PA (31). Activity of PAI-2 was also analyzed by fibrin zymography, following non-denaturing gel electrophoresis (33). Pregnancy plasma was run as a positive control for PAI-2 and was incubated with an antibody to α₂-AP (Dako, UK) prior to electrophoresis.

Analysis of transglutaminase-catalyzed cross-linking. Fibrinogen (1 µM, Kabi, Sweden) was diluted in PBS with or without addition of 200 nM rPAI-2 (Delta Biotechnology, UK) or 200 nM α₂-AP (Biopool, Sweden). Purified guinea pig liver transglutaminase (Sigma, UK) or human plasma FXIIIa (American Diagnostica, USA) was added to the clots at final concentrations of 200 nM or 400 nM. Fibrin formation was initiated by addition of bovine thrombin (0.4 U/ml) and 2.5 mM CaCl₂. Inhibition of transglutaminase activity was achieved by addition of either 200 nM Tridegin (34) (Biopharm UK Ltd), 10 mM EDTA or 80 mM iodoacetamide (IAA), prior to addition of transglutaminases. Peptides were synthesized in-house and were incorporated into clots at a final concentration of 20 µM. The sequences of the peptides were as follows –

Peptide 1 Gly-Phe-Met-Gln-Gln-Ile-Gln-Lys-Gly-Ser
 Peptide 2 Gly-Phe-Met-Ser-Ser-Ile-Ser-Lys-Gly-Ser
 Peptide 3 Gly-Phe-Met-Gln-Ser-Ile-Ser-Lys-Gly-Ser
 Peptide 4 Gly-Phe-Met-Ser-Gln-Ile-Ser-Lys-Gly-Ser
 Peptide 5 Gly-Phe-Met-Ser-Ser-Ile-Gln-Lys-Gly-Ser.

Clots were incubated at 37° C for 1 h, and were then solubilized with an equal volume of 8 M urea, 200 mM Tris, 4% w/v SDS, 40 mM dithiothreitol for 30 min at 37° C. These samples were analyzed by SDS-PAGE on 3-12% acrylamide gels. Proteins were transferred overnight to nitrocellulose membrane (Bio-Blot-NC, Costar, UK) and immunoblotted for either PAI-2 or α₂-AP. Membranes were blocked with 50 mM carbonate/bicarbonate buffer pH 9.6 containing 3% w/v bovine serum albumin. Antigen was detected using a mouse monoclonal antibody to PAI-2 (Biostat, UK) or a rabbit antibody to α₂-AP (Dako, UK), followed by either anti-mouse or anti-rabbit IgG preparation conjugated to alkaline phosphatase. Colour was detected using 200 mM Tris/HCl, 10 mM MgCl₂, pH 9.5 containing 0.6 mM 5'-bromo 4-chloro 3-iodo-phosphate and 0.5 mM nitroblue tetrazolium.

Immunoprecipitation of fibrin clots was carried out by addition of 500 µl 50 mM Tris/HCl, pH 8.0, 500 mM NaCl, 50 mM EDTA, 1% Tween 20 (NET buffer) to disperse the fibrin clot. A mouse monoclonal antibody to fibrin (35) was incubated with samples for 90 min, and 50% v/v protein A Sepharose in NET buffer was rotated at 4° C for 90 min. Centrifugation was employed to collect the antibody-antigen-Sepharose bead complex, and the beads were washed twice with NET buffer and twice with 10 mM Tris, pH 6.8. The Sepharose beads were incubated in 8 M urea, 200 mM Tris, 4% w/v SDS, 40 mM dithiothreitol, and electrophoresed on 3-12% gradient acrylamide gels as above. PAI-2 antigen, cross-linked to the fibrin, was detected using a rabbit IgG to PAI-2 (16). Colour was developed as above.

Results

Inhibition of fibrin clot lysis by recombinant PAI-2. The effect of recombinant PAI-2 on fibrin clot lysis was analyzed using a functional assay. Clots were formed from purified plasminogen and fibrinogen by addition of thrombin and calcium. Lysis was induced using either u-PA

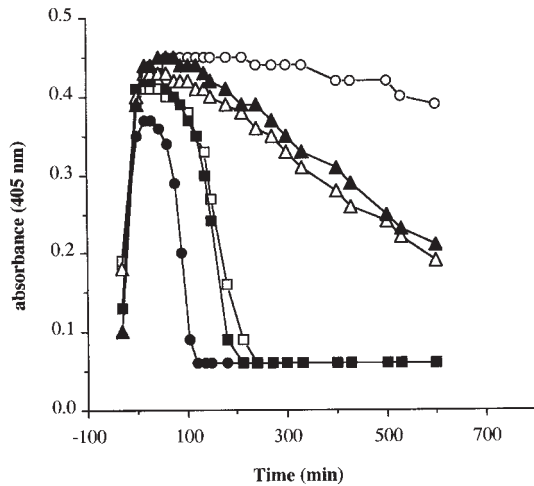


Fig. 1 Inhibition of u-PA-mediated clot lysis by rPAI-2 and rPAI-1. Fibrin clots were allowed to form in a microtitre plate by incubation of 2.94 μ M fibrinogen, 0.24 μ M plasminogen in the presence of 0.4 U/ml thrombin and 5.3 mM CaCl_2 . No u-PA (\circ) or 180 pM u-PA (\bullet) was added to the clots. The inhibitory effect of 60 mU/well rPAI-1 (\square), 60 mU rPAI-2 (\blacksquare), 120 mU rPAI-1 (\triangle) and 120 mU rPAI-2 (\blacktriangle) was monitored by turbidity at 405 nm

(180 pM) or t-PA (20 pM), and the effect of recombinant PAI-2 on lysis was studied. Recombinant PAI-2, and PAI-1 for comparison, were used at 30, 60, 120, and 180 mU/well, the unit of activity being defined by inhibition of u-PA-mediated cleavage of the chromogenic substrate, S-2444 (31). The inhibitory effect of PAI-2 and PAI-1, at 60 and 120 mU/well, on u-PA-mediated clot lysis is shown (Fig. 1). Both PAI-2 and PAI-1 showed a dose-dependent inhibition of lysis. Importantly, PAI-2 was as efficient as PAI-1 in this assay; this was true for all concentrations of PAI-2 and PAI-1 studied. The time for 50% clot lysis on addition of u-PA was 80 min. This time was increased to 110 min in the presence of 30 mU PAI-2 and PAI-1, and 170 min and 180 min in the presence of 60 mU PAI-2 and 60 mU PAI-1, respectively. Addition of 120 mU PAI-2 and 120 mU PAI-1 increased the time for 50% lysis to 510 min and 530 min, while it was greater than 700 min on addition of 180 mU PAI. PAI-2 did not inhibit either single-chain or two-chain t-PA-mediated clot lysis, even at elevated concentrations of 2 μ M PAI-2, but PAI-1 inhibited t-PA induced lysis in a dose-dependent manner (data not shown).

Inhibition of fibrin clot lysis by cellular PAI-2. The effective inhibition of u-PA-induced fibrin clot lysis by recombinant PAI-2 was extended to investigate a cellular source of PAI-2. Human peripheral blood monocytes were studied, activated monocytes being an abundant source of PAI-2 (16). Isolated monocytes were either untreated or stimulated with lipopolysaccharide (LPS), which up-regulates both secreted and intracellular forms of PAI-2. Conditioned medium was removed after 18 h incubation, and monocytes were overlaid with a fibrin clot. Monocytes inhibited u-PA- but not t-PA-induced fibrin clot lysis (Fig. 2A). This inhibition was only seen with activated monocytes, which correlated with an up-regulation in the synthesis of PAI-2. The time for 50% lysis of the clot by t-PA (20 pM) was 50 min in the presence of unstimulated and LPS-stimulated monocytes. U-PA-mediated clot lysis (180 pM NIBSC u-PA) showed a half lysis time of 100 min in the presence of unstimulated monocytes, which increased to 340 min following LPS-stimulation. This increase in lysis time was due to PAI-2, since antibodies to PAI-2 neutralized the inhibitory effect (Fig. 2B). In contrast, antibodies to PAI-1 (Fig. 2B) or normal rabbit

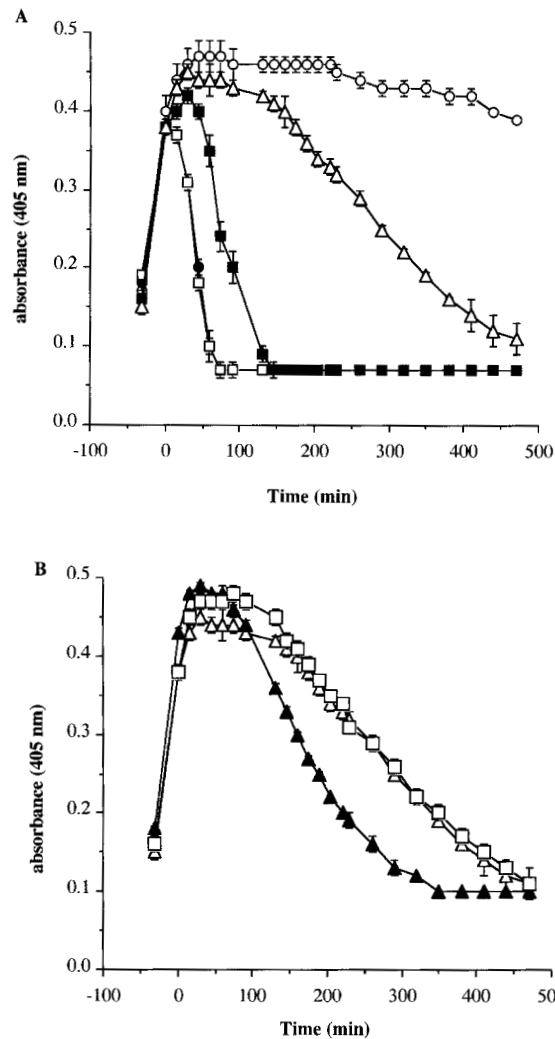


Fig. 2 Peripheral blood monocytes inhibit u-PA-mediated clot lysis. A) Isolated monocytes were either untreated or treated with LPS (10 ng/ml) for 18 h. Conditioned medium was removed, and monocytes were overlaid with 2.94 μ M fibrinogen, 0.24 μ M plasminogen, 0.4 U/ml thrombin and 5.3 mM CaCl_2 . The effect of untreated (\bullet) or LPS-treated (\square) monocytes on lysis by t-PA (20 pM), and the effect of untreated (\blacksquare) or LPS-treated (\triangle) monocytes on lysis by u-PA (180 pM) was investigated. Monocytes were also overlaid with fibrin clots containing no added t-PA or u-PA (\circ). Values represent mean \pm SEM, from triplicate wells of monocytes from a representative donor, the experiment being typical of a total of six. B) The inhibitory effect of LPS-treated monocytes on fibrin lysis by u-PA (180 pM) was investigated further. Fibrin clots contained 250 μ g/ml rabbit IgG to PAI-1 (\square), to PAI-2 (\blacktriangle) or no antibody (\triangle). Values represent mean \pm SEM, from triplicate wells of monocytes from a representative donor, typical of 6 experiments

IgG (data not shown) had no effect on clot lysis. The addition of antibodies to PAI-1 or PAI-2 had no effect on clot lysis by unstimulated monocytes. The data shown here represent monocytes isolated from one donor, since variability is seen between monocyte populations (16). Inhibition of clot lysis by activated monocytes was reproducible on five further occasions. In all cases, activated monocytes inhibited only u-PA-induced lysis and not t-PA-mediated clot lysis, and antibodies to PAI-2 neutralized the inhibitory effect.

Inhibition of fibrin clot lysis by monocyte-derived PAI-2 demonstrated that monocytes had secreted PAI-2, which had penetrated the overlying clot. PAI-2 antigen was analyzed by ELISA in the condi-

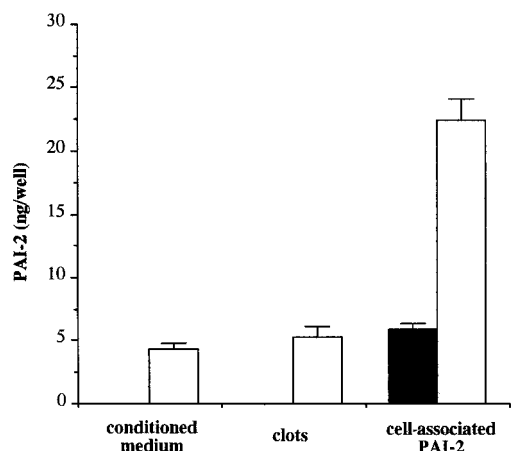


Fig. 3 PAI-2 is secreted by monocytes into the fibrin clot. Isolated monocytes were either untreated (■) or LPS-treated (□) for 18 h. Conditioned medium was removed and monocytes were overlaid with 2.94 μ M fibrinogen, 0.24 μ M plasminogen, 0.4 U/ml thrombin and 5.3 mM CaCl_2 . Lysis of clots was induced by addition of 180 pM u-PA, and absorbance at 405 nm was monitored. The solubilized clots were removed from monocytes, and monocytes were scraped from the microtitre well. PAI-2 (ng/well) was analyzed by ELISA in conditioned medium, in solubilized clots and in monocyte lysates. Values represent mean \pm SEM, from triplicate wells of monocytes from a representative donor, typical of 6 experiments

tioned medium that had been removed prior to the assay, in the solubilized clot, and in the cells that had been scraped from the microtitre plate following the assay. PAI-2 antigen in the conditioned medium of LPS-treated monocytes reached a concentration of 3.8 ng/well following 18 h incubation, and was not detected in the conditioned medium of unstimulated cells (Fig. 3). Similarly, PAI-2 was detectable only in the clots that had been removed from LPS-stimulated monocytes, where it was present at 5.3 ng/well. Cell-associated PAI-2 was detected at higher levels. Unstimulated cells accumulated PAI-2 at 5.9 ng/well, which increased to 22.4 ng/well on LPS treatment. Cell-associated PAI-2 remained the biggest pool over the time course of the clot lysis assay, some 8 h, but a considerable amount (approximately 25%) of PAI-2 was secreted into the clot, where it efficiently inhibited clot lysis. The fibrin clots were also assayed for lactate dehydrogenase (LDH) by colorimetric assay, to evaluate whether the monocytes had undergone cell death, releasing PAI-2 into the fibrin clot. This was not the case, since LDH was not detectable in the solubilized fibrin clot. The clots were also analyzed for the presence of cells by the Trypan blue membrane exclusion stain and microscopy. No monocytes were evident, showing that the cells had not become detached from the tissue culture plate.

PAI-2 is present in human thrombi. Extracts from arterial and venous human thrombi were also examined for the presence of active PAI-2 by fibrin zymography following non-denaturing gel electrophoresis. A zone of inhibitory activity (Fig. 4, track A) was seen in both arterial and venous thrombus extracts. This inhibitory activity was demonstrated to be PAI-2, since only antibodies to PAI-2 neutralized the inhibitory zone (Fig. 4, track D). Antibodies to PAI-1 (Fig. 4, track B), or α_2 -AP (Fig. 4, track C) had no effect. The extract was also incubated with all three antibodies (Track E), and pregnancy plasma was used as a positive control for PAI-2 activity (Track F). PAI-2 antigen was extractable from thrombi over a wide range, 0-87 ng/g tissue, and there was no clear difference between the venous and arterial thrombi in this respect.

Cross-linking of PAI-2 to fibrin by transglutaminases. We investigated whether PAI-2 could be cross-linked to fibrin, and analyzed this phenomenon in a purified system using TTG and FXIII. α_2 -AP is known to be cross-linked to fibrin, in a reaction that is catalyzed by activated FXIII (27), and this was included for comparison. Fibrinogen was clotted by addition of thrombin and calcium chloride, in the presence of recombinant PAI-2 or α_2 -AP and purified TTG or purified FXIII. The formation of cross-linked higher molecular mass species of PAI-2/ α_2 -AP was assessed by SDS-PAGE on 3 to 12% acrylamide gradient gels (mini or maxi gels) and western blotting.

PAI-2 was cross-linked to fibrin and formed higher molecular mass forms of PAI-2 (Fig. 5A). Cross-linking was achieved by either TTG or FXIII, in a dose-dependent manner. The cross-linked forms of PAI-2 were observed at a variety of molecular masses, all greater than 120 kDa (tracks e-h); no PAI-2 was detectable in the stacking gel. Some differences were observed depending on whether TTG or FXIII was used. The formation of these cross-linked products was dependent on the presence of transglutaminase and calcium. It is known that PAI-2 can cross-link/polymerize to itself (23). The use of gradient gels clearly distinguished between cross-linking of PAI-2 to fibrin and cross-linking/polymerization of PAI-2 to itself (note the diffuse band of about 90 kDa in track a-c). Distinct high molecular mass forms of PAI-2 were found only in the presence of fibrin and TG, traces of these bands in track d suggesting that the fibrinogen used was contaminated with FXIII. The intense high molecular mass bands seen in tracks e-h were confirmed as representing PAI-2 cross-linked to fibrin by immunoprecipitation of clots with a monoclonal antibody to fibrin (35) and immunoblotting with antibodies to PAI-2 (data not shown). Cross-linking of α_2 -AP to fibrin was also achieved by TTG or FXIII (Fig. 5B). In contrast, rPAI-1 was not cross-linked to fibrin (data not shown).

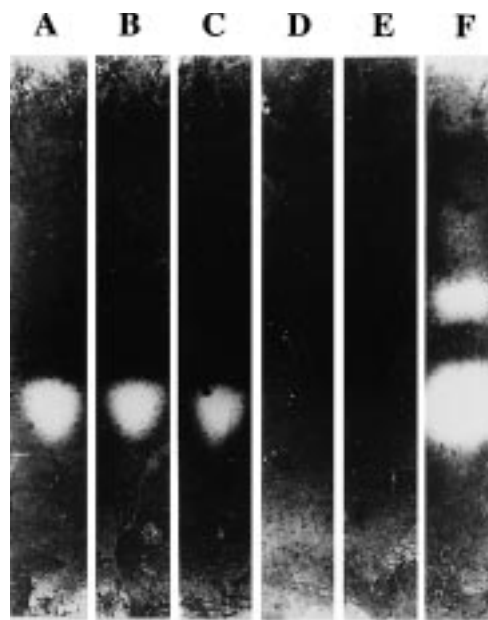


Fig. 4 PAI-2 is present in human thrombi. Extracts of arterial and venous human thrombi were made. The extract (30 μ l per track) was incubated with 100 μ g pre-immune rabbit IgG (A), rabbit IgG to PAI-1 (B), rabbit IgG to α_2 -AP (C), rabbit IgG to PAI-2 (D) or 100 μ g of each antibody (E). Pregnancy plasma was incubated with the rabbit IgG to α_2 -AP (F). Samples were run on a non-denaturing gel, and the gels were overlaid on a fibrin plates, which were lysed with u-PA (0.05 IU/ml)

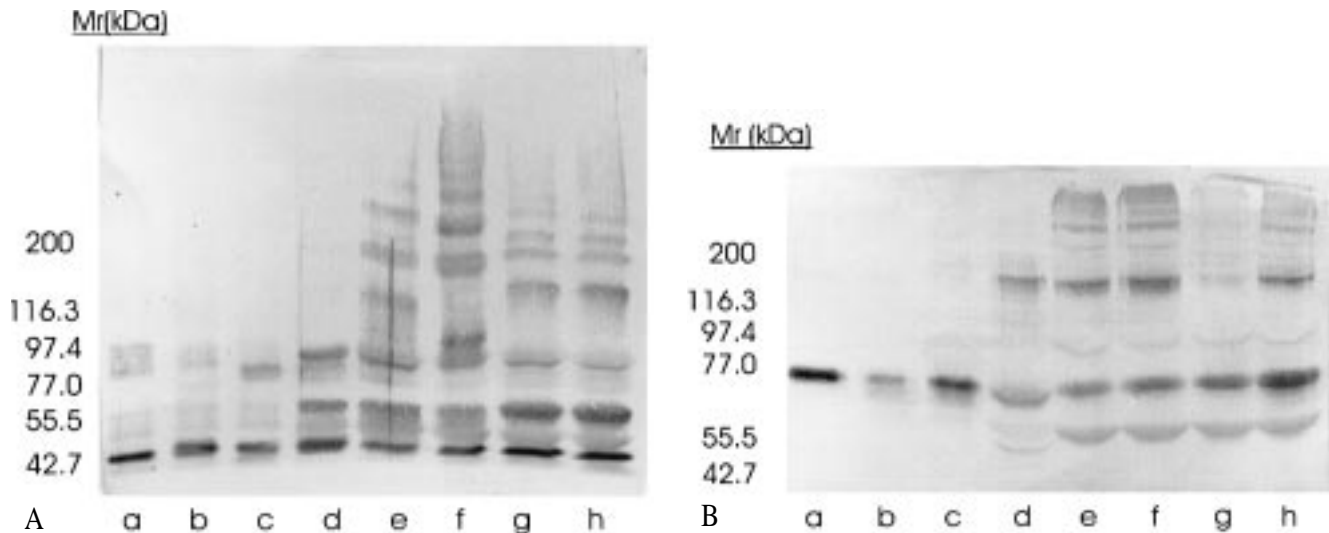


Fig. 5 Cross-linking of PAI-2 and α_2 -AP to fibrin. The effect of TTG and FXIIIa on cross-linking of PAI-2 (A) and α_2 -AP (B) was studied in a purified system containing either fibrinogen (1 μ M), 0.4 U/ml thrombin, 2.5 mM CaCl_2 , 200/400 nM TTG, or 200/400 nM FXIIIa. Samples were incubated for 1 h at 37° C and clots were analyzed by SDS-PAGE on 3-12% acrylamide mini gels, followed by western blotting with antibodies to PAI-2 or α_2 -AP. PAI-2/ α_2 -AP (200 nM), were incubated for 1 h at 37° C with additions as follows: (a) no addition, (b) 200 nM TTG, thrombin and CaCl_2 , (c) 200 nM FXIIIa, thrombin and CaCl_2 , (d) fibrinogen, thrombin and CaCl_2 , (e) fibrinogen, 200 nM TTG, thrombin and CaCl_2 , (f) fibrinogen, 400 nM TTG, thrombin and CaCl_2 , (g) fibrinogen, 200 nM FXIIIa, thrombin and CaCl_2 , (h) fibrinogen, 400 nM FXIIIa, thrombin and CaCl_2

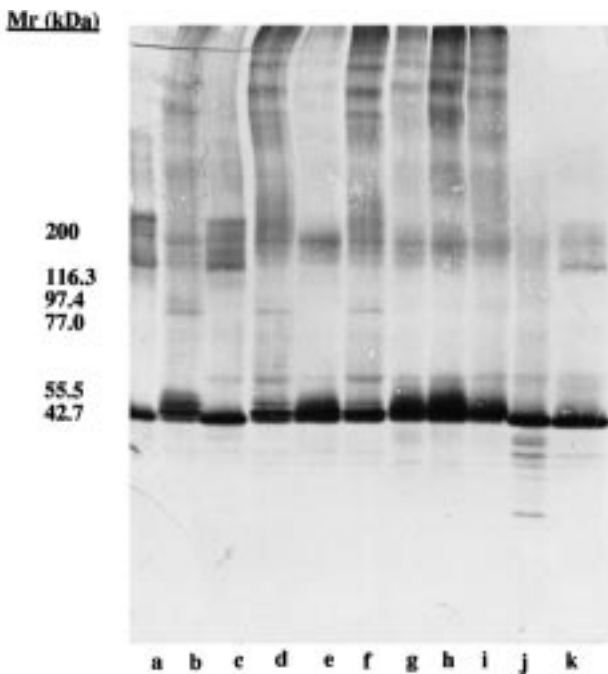


Fig. 6 Mechanism for cross-linking of PAI-2 to fibrin. PAI-2 (200 nM) was incubated alone (a) or in the presence of 200 nM TTG and 2.5 mM CaCl_2 (b). Cross-linking of PAI-2 (200 nM) to fibrin was studied as follows in the presence of fibrinogen (1 μ M), 0.4 U/ml thrombin, 2.5 mM CaCl_2 for 1 h at 37° C. Samples were analyzed by SDS-PAGE on 12% acrylamide maxi gels and western blotting for PAI-2. (c) no further addition, (d) with 200 nM TTG, (e) with 200 nM TTG and 20 μ M peptide 1 (QQIQ), (f) with 200 nM TTG and 20 μ M peptide 2 (SSIS), (g) with 200 nM TTG and 20 μ M peptide 3 (QSIS), (h) with 200 nM TTG and 20 μ M peptide 4 (SQIS), (i) with 200 nM TTG and 20 μ M peptide 5 (SSIQ), (j) with 200 nM TTG and 200 nM Tridegin, (k) with 200 nM TTG and 10 mM EDTA

Mechanism of cross-linking of PAI-2 to fibrin. The mechanism of cross-linking of PAI-2 to fibrin was investigated by studying the involvement of residues 80-90, and in particular the role of Gln 83, 84 and 86, by incorporation of peptides into the cross-linking reaction. Peptide 1 contained all three glutamine residues, and peptide 2 had all glutamine residues replaced by serine residues. Peptides 3 to 5 contained only one glutamine, glutamine 83 or 84 or 86, respectively. Peptide 1 inhibited cross-linking of PAI-2 to fibrin, competing with the cross-linking site on PAI-2 (Fig. 6, track e). Peptide 2, with no glutamines, acted as a control peptide, and had no effect on cross-linking of PAI-2 to fibrin (track f). Peptide 3, which contained glutamine 83, inhibited cross-linking (track g). In contrast, glutamine 84 did not affect cross-linking of PAI-2 to fibrin (peptide 4, track h). Glutamine 86 was also involved in cross-linking, since peptide 5 competed for cross-linking of PAI-2 (track i). This demonstrated that glutamines 83 and 86 are important for cross-linking of PAI-2 to fibrin, but inhibition of cross-linking was most effective in the presence of the peptide containing all three glutamine residues.

The dependence of cross-linking on transglutaminase activity was investigated using Tridegin, which is a specific inhibitor of activated FXIII and tissue TG. Tridegin has been isolated from the leech, *Haementeria ghilianii* (34) and was kindly supplied by Biopharm UK Ltd. Tridegin was incorporated into the system at the same final molar concentration as transglutaminases (200 nM). Tridegin inhibited cross-linking of PAI-2 to fibrin (Fig. 6, track j). Transglutaminases require an active-site cysteine residue and calcium for activity, and therefore general inhibition of TG can be achieved by the use of alkylating and chelating agents, IAA and EDTA respectively. Both IAA (data not shown) and EDTA (Fig. 6, track k) inhibited cross-linking as did Tridegin (Fig. 6). Cross-linking of α_2 -AP to fibrin was similarly inhibited by Tridegin, IAA and EDTA (data not shown).

Effect of cross-linking on activity of PAI-2. We have shown that rPAI-2 was cross-linked to fibrin and that monocyte-derived PAI-2 efficiently inhibited fibrin clot lysis. This was extended by studying the effect of cross-linking on activity of rPAI-2, using the purified system, in which 200 nM TTG was added to fibrin in the presence and absence of rPAI-2 at 60, 120, and 180 mU/well. Addition of TTG had no effect on u-PA-mediated clot lysis, and the time for half lysis was 100 min whether or not TTG was present (Fig. 7). PAI-2 (60 mU/well) inhibited clot lysis and the time for half lysis was increased to 250 min. No effect of TTG was seen and the time for half lysis remained at 250 min, showing that cross-linking did not affect the activity of rPAI-2. Addition of TTG similarly had no effect on the inhibitory activity of rPAI-2 at 120 or 180 mU/well (data not shown). Further support for the retention of PAI-2 activity after cross-linking comes from assay of PAI-2 inhibition of u-PA, measured with the chromogenic substrate S2444. We found no effect on PAI-2 activity following the inclusion of TTG or FXIII in the presence of fibrin or fibrinogen (data not shown).

Monocytes were overlaid with fibrin clots, which were subsequently analyzed for activity of PAI-2, by fibrin zymography following non-denaturing gel electrophoresis, a semi-quantitative technique (33). The activity of PAI-2 in the clots was compared to the activity of PAI-2 in conditioned medium of monocytes and in pregnancy plasma, which has been previously studied (36). Equivalent amounts of PAI-2 antigen (2 ng) from conditioned medium and fibrin clots were electrophoresed, and both sources of PAI-2 were equally active (Fig. 8). PAI-2 in conditioned medium electrophoresed alongside the fast migrating band of PAI-2 seen in pregnancy plasma. These inhibitory bands were shown to be PAI-2 by incorporation of a rabbit antibody to PAI-2, which neutralized activity (data not shown). It was interesting to note that monocyte-derived PAI-2 in the fibrin clot migrated slightly differently to PAI-2 in conditioned medium and pregnancy plasma. It was also strikingly efficient in inhibiting clot lysis, such that the gel had to be incubated for an additional 24 h for the opaque zone to become clear, by which time the slow band of PAI-2 in pregnancy plasma had almost disappeared.

Discussion

This study has shown that monocyte PAI-2 retains activity after being cross-linked to fibrin by the two major transglutaminases, tissue TG and FXIII. PAI-2 is the second serpin that is localized to the fibrin surface in this manner, the well-known example being α_2 -AP (26). In this situation, monocyte PAI-2 acts as an efficient inhibitor of u-PA-mediated fibrin clot lysis.

Both recombinant PAI-2 and monocyte-derived PAI-2 effectively inhibited u-PA-mediated fibrin clot lysis, whereas it had no effect on t-PA. This agrees with a previous report that fibrin-bound t-PA, but not poly-D-lysine-stimulated t-PA, was resistant to inactivation by minactivin (PAI-2) (37). The lack of inhibition of t-PA may reflect protection of t-PA activity by fibrin (37). In the absence of fibrin, PAI-2 can inhibit two-chain t-PA (12), whereas in this study it had no effect on either single-chain or two-chain t-PA. In contrast, its inhibition of u-PA was comparable to that of PAI-1, over the time course of a fibrin clot lysis assay, despite its being an order of magnitude poorer an inhibitor of u-PA on the basis of its second order rate constant (12).

The inhibition of u-PA by PAI-2 argues for a role for PAI-2 in local environments and the association of u-PA and PAI-2 with cell-associated fibrinolysis (18). u-PA has been implicated in migration of cells and extracellular matrix remodelling, and u-PA binds to its cellular receptor, u-PAR (39). Accumulation of u-PA in the diseased vessel wall has been shown in studies in the mouse and human (40,5). There-

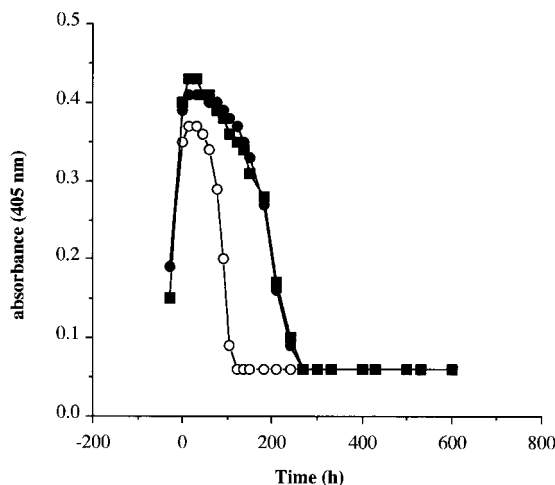


Fig. 7 Effect of transglutaminase on activity of PAI-2. Fibrin clots were allowed to form in a microtitre plate by incubation of 2.94 μ M fibrinogen, 0.24 μ M plasminogen in the presence of 0.4 U/ml thrombin and 5.3 mM CaCl_2 . Lysis was achieved by addition of 180 pM u-PA (\circ). The inhibitory effect of 60 mU/well rPAI-2 (\bullet) and 60 mU/well rPAI-2 in the presence of 200 nM TTG (\blacksquare) was investigated by monitoring the absorbance at 405 nm

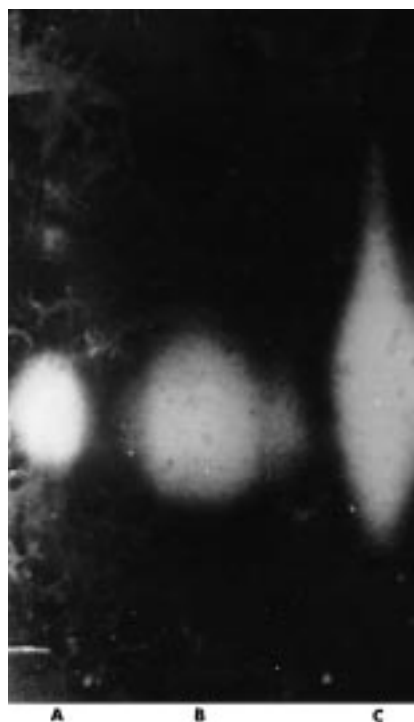


Fig. 8 Activity of PAI-2 secreted by monocytes into fibrin clot. Isolated monocytes were treated with 10 ng/ml LPS for 18 h and the conditioned medium was removed. Monocytes were overlaid with 2.94 μ M fibrinogen, 0.24 μ M plasminogen, 0.4 U/ml thrombin and 5.3 mM CaCl_2 and lysis was induced by addition of 180 pM u-PA. Solubilized clots were collected. Samples of conditioned medium (B) and clot (C), corresponding to 2 ng PAI-2 measured by ELISA, was run on non-denaturing gels and activity of PAI-2 was analyzed by fibrin zymography. Pregnancy plasma was run as a positive control for PAI-2 (A)

fore, PAI-2 is likely to contribute to persistence of fibrin in environments such as the atherosclerotic plaque. It has recently been observed that PAI-2 antigen is indeed present within the vessel wall (7). Here, we demonstrate the presence of active PAI-2 in extracts of human thrombi, which suggests that PAI-2 may also have a role in protecting thrombi from dissolution. PAI-2 antigen was extractable from both arterial and venous thrombi, ranging from 0 to 87 ng/g of tissue. There was no correlation between the concentration of PAI-2 and the source of the thrombus, whether arterial or venous. It is possible that only a proportion of the thrombus PAI-2 was extractable as a result of the cross-linking that this study demonstrates. In contrast, PAI-1 antigen was extractable from the same thrombi in the range of 40–7700 ng/g tissue (6). The cellular source of PAI-2 in the thrombus is as yet unknown, but it is possible that activated monocytes secrete active PAI-2 into the thrombus.

Cross-linking of PAI-2 to fibrin was achieved by TTG or activated FXIII. These two enzymes show different specificities, with TTG preferentially mediating cross-linking of the α -chains of fibrinogen, whereas activated FXIII cross-links γ -chains (28). The distribution of TTG and FXIII is different, and TTG is constitutively expressed by endothelial cells, macrophages; expression of TTG is elevated during wound healing (41). FXIII is found in plasma at 700 nM and is also detected in monocytes and platelets, which allows localization of FXIII at the site of thrombus formation (25). Cross-linking of PAI-2 to fibrin was compared with that of α_2 -AP, since this inhibitor plays a significant role in protection of a fibrin clot from lysis (27). Both FXIII and TTG catalyse cross-linking of α_2 -AP to the α -chain of fibrinogen, via a glutamine residue at the N-terminus of α_2 -AP (42). Cross-linking of PAI-2 to fibrin did not affect the activity of PAI-2, in agreement with data on PAI-2 cross-linked to cellular membranes (23). The loop that contains glutamine residues 83, 84 and 86 is distant from the reactive centre (22), consistent with the lack of effect of cross-linking on activity. PAI-2 that was secreted by monocytes into an overlying clot was also active. It is not yet known if this material was cross-linked, but it was interesting to note that PAI-2 from the fibrin clot migrated differently to PAI-2 in conditioned medium or in pregnancy plasma. Monocytes can synthesize both TTG and FXIII; these cells have secreted and intracellular pools of both enzymes (25).

PAI-2 was found to cross-link to fibrin via glutamine residues present on the interhelical loop (residues 66 to 98) and we demonstrated that not every glutamine residue played an equal role as a donor in the cross-linking reaction. Competition by peptides showed that glutamines 83 and 86 were important in cross-linking, glutamine 83 being the principal residue involved. Glutamine 84 did not appear to be necessary, perhaps reflecting steric hindrance and proximity to glutamine 83. It is interesting that glutamines 83 and 86 are conserved in PAI-2 from other species, for instance in rat (22).

Cross-linking of both PAI-2 and α_2 -AP was inhibited by Tridegin, a novel specific inhibitor of FXIII and TTG isolated from the leech (34). Tridegin and hirudin, a specific inhibitor of thrombin (43), inhibit fibrin clot cross-linking and fibrin clot formation, respectively, which presumably allows the leech to feed easily on host blood. Tridegin has been found to enhance fibrinolysis by inhibition of cross-linking between fibrin strands (44). Tridegin was a useful inhibitor of transglutaminase activity, and gave similar results to inhibition by the general inhibitors of TG, iodoacetamide and EDTA (25).

Monocytes represent a physiological source of PAI-2, and these cells not only accumulate PAI-2 intracellularly but also secrete PAI-2 (16). Secretion of PAI-2 by peripheral blood monocytes occurs via an ER-Golgi independent pathway, which results in an abundance of

47 kDa non-glycosylated PAI-2 in the medium surrounding monocytes (17). This is in contrast to other cell types such as U937 cells, where PAI-2 is secreted as a 60 kDa glycosylated PAI-2 (45). We found that secretion of PAI-2 by monocytes into the overlying fibrin clot occurred rapidly, and PAI-2 penetrated the fibrin clot and inhibited fibrin clot lysis within the time course of the clot lysis assay. Antibodies to PAI-2 neutralized the inhibitory effect, but this neutralization was not complete even at a 2000-fold excess of antibody concentration over PAI-2 antigen. It is possible that PAI-2 cross-linked to fibrin is not readily accessible to antibody. It is interesting that non-glycosylated PAI-2 is secreted by monocytes, since it has been postulated that glycosylation of PAI-2 at asparagine 75 prevents cross-linking of PAI-2 via glutamine residues 83 to 86 (12). Non-glycosylated PAI-2 secreted by monocytes would therefore be available for cross-linking by transglutaminases.

PAI-2 synthesis by monocytes is up-regulated by both thrombin and D-dimer (16, 46). Thrombin, which is present in the fibrin clot, may act to maintain synthesis and secretion of PAI-2 by monocytes, acting as a positive feedback loop. D-dimer is produced as fibrin is degraded and this mechanism may also act to mediate persistence of fibrin by an up-regulation in PAI-2 by monocytes.

PAI-2 production by monocytes is an efficient inhibitor of u-PA-mediated fibrin clot lysis. The physiological relevance of this is currently under investigation, since u-PA is highly expressed in the atherosclerotic plaque (5). It is becoming increasingly evident that PAI-2 is present in many situations, including the atherosclerotic plaque and thrombi, where monocytes are a major cell type. Cross-linking of PAI-2 serves to localize activity at the fibrin surface, where it may contribute to persistence of fibrin, either within the fibrin clot or inflammatory lesions.

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