Less pronounced enhancement of thrombin-dependent inactivation of plasminogen activator inhibitor type 1 by low molecular weight heparin compared with unfractionated heparin

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Summary
Plasminogen activator inhibitor type 1 (PAI-1), the primary inhibitor of plasminogen activators, also forms high molecular weight complexes with either thrombin or factor Xa (FXa) in the presence of heparin, resulting in the loss of mutual activities of enzyme and inhibitor. We have proposed that the inactivation of PAI-1 by these activated coagulation factors is one of the mechanisms responsible for coagulation-associated enhancement of fibrinolysis. In the present study, we compared the effects of low molecular weight (LMW)- and unfractionated-heparin on the interaction between PAI-1 and either thrombin or FXa. Both types of heparin enhanced the inhibition of thrombin activity by PAI-1 with a bell-shaped pattern, though the magnitude of the enhancement was significantly weaker with LMW-heparin. In FXa inhibition by PAI-1, only unfractionated-heparin enhanced the inhibition. In the presence of vitronectin (Vn), the inhibition of thrombin and FXa by PAI-1 was further promoted by both types of heparin but to a significantly lesser extent with LMW-heparin. We then analyzed the possible enhancing effect of heparin on tissue plasminogen activator (tPA)-induced fibrinolysis. As a consequence of thrombin-dependent inactivation of PAI-1, tPA-induced fibrin clot lysis time in the presence of PAI-1 was shortened by unfractionated-heparin as well as by LMW-heparin with lesser extent, which was further enhanced by Vn. Less pronounced enhancement of complex formation between thrombin and PAI-1 by LMW-heparin appeared to be directly related to the weaker potential of LMW-heparin in enhancing fibrinolysis and accelerating hemorrhagic tendency via neutralization of PAI-1 activity.

Keywords
Plasminogen activator inhibitor type 1, low molecular weight heparin, unfractionated heparin, thrombin, vitronectin

Thromb Haemost 2006; 95: 637–42

Introduction
Plasminogen activator inhibitor type 1 (PAI-1) belongs to the serine protease inhibitor superfamily (SERPINS) (1) that includes a large number of protease inhibitors in plasma. In the vasculature, tissue type plasminogen activator (tPA) is the target protease, and its activity as well as total fibrinolytic activity in plasma are determined by the balance between tPA and PAI-1 (2). Their imbalance is directly related to either over-expression or impairment of fibrinolytic activity. As well as complexing with plasminogen activators (PAs), PAI-1 also forms complexes with other active serine proteases involved in the coagulation cascade, including factor XIa (FXIa), kallikrein, FXIIa (3), thrombin (4, 5), and Ca²⁺-bound FXa (6), especially in the presence of either heparin or vitronectin (Vn). As a result of complex formation with these active coagulation factors, PAI-1 loses its specific activity (7), resulting in the enhancement of fibrinolysis due to the relative increase in tPA over PAI-1. We have proposed (8) that this is one of the mechanisms involved in so-called coagulation-associated enhancement of fibrinolysis (9), which plays an essential role in maintaining vascular patency by limiting over-accumulation of fibrin clots.

Over-expression of fibrinolytic activity in disseminated intravascular coagulation (DIC) is a pathological phenotype of this event. DIC occurs when the coagulation cascade is activated systemically under various pathological conditions, and numerous microthrombi would be formed in the minute blood vessels in the whole body. In addition to so-called consumption-coagulopathy, uncontrollable extensive enhancement of fibrinolysis to dissolve multiple thrombi is an important mechanism to induce bleeding.
tendency. Several different mechanisms have been proposed for the coagulation-associated enhancement of fibrinolysis and bleeding tendency in DIC (8, 9).

The principle of the treatment of DIC is anticoagulation, and heparin has been commonly used because of its enhancing effect of anti-thrombin activity. To reduce the hemorrhagic side effects of heparin in the treatment of DIC, low molecular weight (LMW) heparin (molecular weight: 4,400–5,600), which is obtained by means of nitrous acid, has become more frequently used. The advantage of the lower bleeding tendency of LMW-heparin seems to be due to its weaker effect on the inactivation of thrombin by anti-thrombin (10, 11). It has been ascertained that LMW-heparin with molecular weights of less than about 5,000 does not enhance the inactivation of thrombin, FIXa and FXIIa by anti-thrombin, but still retains the enhancing ability to inactivate FXa, FXIIa and kallikrein (12). Therefore, suppression of the activity of FXa, which is equivalent to that of unfractionated heparin, is the major target in LMW-heparin therapy.

In the present study, we compared the effects of LMW- and unfractionated-heparin on the interaction between PAI-1 and either thrombin or Ca2+-bound FXa, and analyzed their possibly different effects on the expression of tPA-dependent fibrinolytic activity.

**Materials and methods**

**Materials**

The cultivation of bacteria expressing human PAI-1 and the purification of active non-glycosylated recombinant PAI-1 (rPAI-1) has been previously described (13). Glu-plasminogen was prepared by affinity chromatography using lysine-Sepharose from freshly frozen plasma. Human fibrinogen was purchased from Enzyme Research Laboratory (South Bend, IN, USA), and trace amounts of contaminated plasminogen and plasmin were removed by passing through lysine-Sepharose. After treatment with 5 mM (final conc.) of phenyl-methyl-sulphonyl-fluoride (PMSF; purchased from Sigma, St. Louis, MO, USA), the material was dialyzed exhaustively against 50 mM 4-(2-hydroxyethyl)-piperazine methanesulfonic acid (Hepes) buffer containing 100 mM NaCl. Single chain tPA (sctPA) was kindly provided by Daiichi Pharmaceutical Co. (Osaka, Japan). Human α-thrombin and FXa were purchased from Mitsubishi Welpharma (Osaka, Japan) and from Enzyme Research Laboratory, respectively, and their active site concentrations were determined by titration with MUGB (14). The following materials were purchased from the indicated sources: chromogenic substrates for FXa (S-2222: benzoyl-Ile-Glu-Gly-Arg-p-nitroanilide-HCl) and for thrombin (S-2238: H-D-Phe-Arg-p-nitroanilide-HCl) from Daidichi Pure Chemicals Co., Ltd. (Tokyo, Japan), human Vn (purified from plasma) from Promega Corporation (Madison, WI, USA), unfractionated-heparin sodium from Takeda Chemical Industries Ltd. (Osaka, Japan), and LMW-heparin sodium from Kissei Pharmaceutical Co., Ltd. (Matsumoto, Japan).

**Measurement of FXa and thrombin activity**

rPAI-1 (40 nM) was incubated with either FXa (0.1 nM) or thrombin (0.2 nM) in the presence of LMW- or unfractionated heparin (0–100 u/ml) in the presence of CaCl2 (5 mM) at 37°C for 15 min in a total volume of 100 µl of 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 0.02% Tween 80 and 0.1% BSA. When required, vitronectin (0–80 nM) was placed in the reaction mixture. After incubation, 5 µl of either 4 mM S2222 or S2238 was added to the reaction mixture and the residual activity of FXa or thrombin was determined from the slope of the linear increase in absorbance at 405 nm versus time (6).

**Analyses of the complex formation between biotin-labeled PAI-1 and either thrombin or FXa**

rPAI-1 was labeled by biotin using a commercially available kit (ECL protein biotinylation module; Amersham Life Science, Buckinghamshire, England) according to the manufacturer's
recommended procedure with slight modifications (15). After an incubation of biotin-labeled rPAI-1 (approximately 40 nM) with either thrombin (400 nM) or FXa (400 nM) in the presence of 10 mM CaCl2 and heparin (1 U/ml) for 60 min at 37 °C, the reaction was stopped by the addition of sample buffer, and the mixture was subjected to 10 % SDS-PAGE. Biotin-labeled rPAI-1-related bands were detected with use of a Streptavidin-HRP conjugate (Amersham Life Science, Buckinghamshire, England) and enhanced chemiluminescence Western blotting detector reagents (ECL™, Amersham Life Science, Buckinghamshire, England) after transblotting onto nitrocellulose membranes.

**Measurement of tPA-induced fibrin clot lysis time**

A 96-well microtiter plate was used for the clot lysis assay. In total, 2 µM of fibrinogen, 0.5 µM of Glu-plasminogen, 5 mM CaCl2, 0.1 nM sctPA, and 4 nM rPAI-1 (when necessary) were added to individual wells, and the clot formation was initiated with 50 nM of human thrombin. Either unfractionated or LMW-heparin was placed in the wells prior to the addition of thrombin. When required, Vn was also placed in the reaction mixture at 0, 20, 40 and 80 nM, together with a fixed concentration of heparin (1 u/ml). Liquid paraffin was then overlaid to prevent the fibrin clot from drying. Absorbance at 405 nm in each well maintained at 37°C was measured every 30 min for up to 50 h, employing an automatic microtiter plate reader (Plate Analyzer ETY-300 TOYO, Tokyo, Japan). The absorbance data were plotted against reaction time, and the clot lysis time was determined as the time giving the midpoint between the maximum and minimum absorbance values (7).

**Statistics**

The data are expressed as mean ± standard error of the mean (SEM) and were statistically evaluated by analysis of variance (ANOVA), followed by the Newman–Keuls-Student test. In the case of comparison between two groups, the analysis was performed by unpaired Student’s t-test; p < 0.05 was considered to be significant.

**Results**

**Effects of unfractionated and LMW-heparin on the inhibition of Ca<sup>2+</sup>-bound FXa activity by rPAI-1**

After incubation of FXa with rPAI-1 in the presence of different concentrations of either unfractionated or LMW-heparin, the residual activity of FXa was assessed by the hydrolysis of the supplemented S2238. As was reported before, unfractionated-heparin enhanced the inhibition of FXa activity by rPAI-1 in the concentration range 1–30 u/ml with a bell-shaped pattern, showing approximately 27.1% inhibition at 10 u/ml of unfractionated-heparin (Fig. 1). LMW-heparin, however, did not enhance the inhibition.

**Effects of unfractionated and LMW-heparin on inhibition of thrombin activity by rPAI-1**

After incubation of thrombin with rPAI-1 in the presence of different concentrations of either unfractionated or LMW-heparin, the residual activity of thrombin was assessed by the hydrolysis of supplemented S2238. Both unfractionated and LMW-heparin significantly enhanced the inhibition of thrombin activity by rPAI-1 in the concentration range 0.01–100 u/ml with a bell-shaped pattern. The enhancement by LMW-heparin, however, was significantly less than that by unfractionated heparin in the concentration range 0.1–100 u/ml. The maximum inhibitions by unfractionated and LMW-heparin were 80% and 21%, respectively, at a dose of 3 u/ml (Fig. 2).
Effect of unfractionated or LMW-heparin on inhibition of Ca\textsuperscript{2+}-bound FXa by PAI-1 in the presence of Vn

Vn also accelerates PAI-1-dependent inhibition of FXa and thrombin (7). We analyzed the possible cooperative effect of Vn and either unfractionated or LMW-heparin on the inhibition of FXa by PAI-1. In the presence of 40 and 80 nM of Vn, the inhibition of Ca\textsuperscript{2+}-bound FXa (0.1 nM) by 40 nM of rPAI-1 was significantly promoted by 10 u/ml of both types of heparin (Fig. 3), though the effect was more prominent with unfractionated heparin than LMW-heparin. These molecules did not inhibit FXa activity in the absence of rPAI-1.

Effects of unfractionated or LMW-heparin on inhibition of thrombin by PAI-1 in the presence of Vn

We analyzed the possible cooperative effect of Vn and either unfractionated or LMW-heparin on the inhibition of thrombin by PAI-1. Vn-dependent enhancement of thrombin (0.2 nM) inhibition by rPAI-1 was further enhanced by both types of heparin (Fig. 4). The enhancement was more prominent in unfractionated heparin than LMW-heparin.

Effects of unfractionated and LMW-heparin on the interaction between PAI-1 and either thrombin or FXa

Biotin-labeled rPAI-1 formed high molecular weight complex with thrombin, and only a small fraction remained as a free form (Fig. 5, upper panel), suggesting that PAI-1 lost its specific activity after interaction with thrombin as reported before (16). Heparin enhanced the interaction between thrombin and PAI-1, and the amount of remaining free PAI-1 was least when unfractionated-heparin was added. When biotin-labeled rPAI-1 was incubated with FXa, the amount of free form also decreased, especially in the presence of unfractionated heparin (Fig. 5, lower panel). In this case, however, bands of high molecular weight forms were not apparent, and only a cleaved low molecular weight form of biotin-labeled rPAI-1 and a degraded form of high molecular weight complex between FXa and PAI-1 were observed especially in the presence of unfractionated heparin. The amount of free form was least again when unfractionated heparin was added.

Effects of unfractionated and LMW-heparin on the shortening of tPA-induced fibrin clot lysis time by thrombin in the presence of PAI-1

Both unfractionated and LMW-heparin enhanced the shortening of tPA-induced fibrin clot lysis time by thrombin in the presence of rPAI-1 (4 nM) with a bell-shaped pattern. The enhancement by LMW-heparin was significantly less prominent than that by unfractionated heparin. The maximum enhancements by unfractionated and LMW-heparin were 68.3% and 32.3%, respectively, at a dose of 1 u/ml (Fig. 6). Vn further shortened the tPA-induced fibrin clot lysis time in the presence of rPAI-1, both in the presence or absence of heparin (Fig. 7). These results are supported by our data that both Vn and heparin additively enhanced thrombin-PAI-1 interaction (Fig. 4). The effects were more prominent in the case of unfractionated heparin.

In the absence of rPAI-1, unfractionated heparin but not LMW-heparin slightly shortened the tPA induced fibrin clot lysis time (Fig. 8), most likely by enhancing tPA catalyzed plasminogen activation (17). Such enhancement by heparin of tPA catalyzed plasminogen activation seems to be the reason why the response curve in fibrin clot lysis time (Fig. 6) is shifted to the left compared to that in thrombin inactivation (Fig. 2).
Discussion

In the present study, we demonstrated that unfractionated heparin potentiated the inhibition of activities of both thrombin and Ca$^{2+}$-bound FXa by PAI-1, both in the presence and absence of Vn. Furthermore, unfractionated heparin enhanced the thrombin-dependent shortening of tPA-induced fibrin clot lysis time in the presence of PAI-1. These effects were less prominent in the case of LMW-heparin.

Over-expression of fibrinolytic activity following systemic micro-thrombus formation, known as DIC, is a pathological phenotype of coagulation-associated fibrinolysis (9). Several different mechanisms are suggested for coagulation-associated enhancement of fibrinolysis. We propose that modification of the well-controlled balance between tPA and PAI-1 by activated coagulation factors is one of them (8). This is based on the fact that both tPA and PAI-1 exist as active forms in plasma, whereas the activity and total fibrinolytic activity are logically determined by the balance between their plasma molar-concentrations. In a purified system, we demonstrated that both Ca$^{2+}$-bound FXa and thrombin (7, 16) enhance fibrinolysis by inactivating PAI-1. Such enhancement of fibrinolysis as a consequence of PAI-1 inactivation has also been demonstrated using activated protein C (18), neutrophil elastase (19), contact phase coagulation factors (3) and subtilisin (20).

PAI-1 binds to heparin through its positively charged residues of Arg115, Arg118 (21), Arg76, Lys80 and Lys88 (22) and acquires its inhibitory activity towards thrombin. We have also shown that high molecular weight complex formation between PAI-1 and either Ca$^{2+}$-bound FXa or thrombin was potentiated by both unfractionated heparin and Vn (7). As is shown in the present study, unfractionated heparin and LMW-heparin have different effects on the interaction between PAI-1 and either thrombin or factor Xa. Inactivation of Ca$^{2+}$-bound FXa activity by PAI-1 was potentiated by unfractionated heparin, which was further enhanced by Vn. In contrast, LMW-heparin had no effect on the inactivation of Ca$^{2+}$-bound FXa activity by PAI-1, and only a weak inhibition was observed in the presence of Vn. The inhibition of thrombin activity by PAI-1 was potentiated by both unfractionated and LMW-heparin and was further enhanced by Vn, though the magnitude of the enhancement was again lower with LMW-heparin than with unfractionated-heparin. As a consequence of the interaction between PAI-1 and either thrombin or FXa, most of PAI-1 lost its specific activity and only a fraction of PAI-1 remained as active free form, especially when unfractionated heparin existed. These results were also well conserved in the tPA-induced fibrin clot lysis experiment. The shortening of tPA-induced fibrin clot lysis time by thrombin in the presence of PAI-1 was enhanced by both unfractionated and LMW-heparin, though the magnitude of the shortening was much greater with unfractionated heparin. These results indicate that LMW-heparin has a less pronounced effect in enhancing fibrinolysis by potentiating the inactivation of PAI-1 by activated coagulation factors. A template mechanism is suggested for heparin and Vn to enhance the interaction between PAI-1 and both Ca$^{2+}$-bound FXa and thrombin (23). In the present study, both unfractionated and LMW-heparin potentiated the neutralization of PAI-1 by thrombin in a bell-shaped activity-concentration curve, which suggests that these molecules function as a template (24, 25). In the case of anti-thrombin, however, LMW-heparin modifies the conformation of anti-thrombin and enhances only the inhibition of factor Xa but not the inhibition of thrombin, which is opposite to what is seen in the present study with PAI-1. A possible difference in conformational alteration in both quality and quantity between anti-thrombin and PAI-1 after binding to heparin (26) may be the reason for such discrepancy, though the precise mechanism has not been clarified.

The principle of the treatment for DIC is anticoagulation, and generally unfractionated heparin used to be administered as an

![Figure 7: Effect of Vn and unfractionated or LMW-heparin on thrombin-dependent shortening of tPA induced fibrin clot lysis time in the presence of rPAI-1.](image)

![Figure 8: Effect of unfractionated or LMW-heparin on thrombin-dependent shortening of tPA induced fibrin clot lysis time in the absence of rPAI-1.](image)
References


Nakamura, et al.: Unfractionated heparin enhances fibrinolysis more strongly than LMW heparin