Highly stable plasminogen activator inhibitor type one (VLHL PAI-1) protects fibrin clots from tissue plasminogen activator-mediated fibrinolysis

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Abstract. Plasminogen activator inhibitor-1 (PAI-1) is the major specific inhibitor of tissue-type plasminogen activator (tPA) which mediates fibrin clot lysis through activation of plasminogen. Wild-type-PAI-1 (wPAI-1) is rapidly converted to the latent form (half-life of ~2 h) and loses its ability to inhibit tPA. We developed a very long half-life PAI-1 (VLHL PAI-1), a recombinant protein with a half-life >700 h compared with wPAI-1. In this study, VLHL PAI-1 was assessed for its ability to inhibit clot lysis in vitro. Clot formation was initiated in normal plasma supplemented with tPA by the addition of either tissue factor or human recombinant FVIIa. Clot lysis time, monitored turbidimetrically in a microtiter plate reader, was determined at various concentrations of wPAI-1 and VLHL PAI-1. Both wPAI-1 and VLHL PAI-1 caused a significant increase in clot lysis time, although the latter was somewhat less effective at lower concentrations. The VLHL PAI-1, but not wPAI-1, maintained its anti-fibrinolytic activity after preincubation overnight at 37˚C. These studies demonstrate that VLHL PAI-1 is an effective inhibitor of fibrin clot degradation. Due to the high stability of VLHL PAI-1 compared with wPAI-1, this novel inhibitor of tPA-mediated fibrinolysis may have therapeutic applications for treating surgical and trauma patients when used directly or in conjunction with the procoagulant recombinant FVIIa.

Introduction

Hemostasis depends on a delicate balance of the coagulation and fibrinolytic systems. An abnormality in either system can lead to excessive bleeding or clotting. The blood coagulation system minimizes blood loss at the site of injury by generating a plasmin-mediated insoluble fibrin clot. Following blood vessel repair, fibrinolytic enzymes dissolve the clot to restore blood flow. Fibrinolysis is finely regulated by the balance between tissue plasminogen activator (tPA), which activates plasminogen to plasmin, plasminogen activator inhibitor type 1 (PAI-1), which inhibits tPA (1), and α(2)-antiplasmin [(α(2)AP)], which inhibits plasmin. Thus, the use of potent blood clotting agents without systemic activation of the coagulation cascade can be beneficial for patients suffering uncontrolled bleeding from conditions such as hemorrhagic disorders or severe trauma. Hemorrhage is a major cause of death of victims of accident- or battlefield-related traumas (2,3). Recombinant factor VIIa (rFVIIa) is one of the more effective treatments for controlling hemorrhage in hemophilia. It has also been used to treat massive bleeding in trauma patients. According to Martinowitz et al (4) and Martinowitz and Michaelson (5), controlled animal trials, small case series, and anecdotal case reports suggest that the use of rFVIIa may slow and control massive bleeding during trauma, and hence prolong survival and reduce mortality. Furthermore, the survival rate of patients suffering from uncontrolled bleeding treated with rFVIIa was 61% (22/36) and was favorable when compared with a published series of similar, or less severe, trauma patients (range 30-57%) (5). This effect is enhanced if the clot is protected from tissue plasminogen activator-induced fibrinolysis. In several studies it has been shown that PAI-1 protects fibrin clots from tPA-mediated plasmin degradation in a dose-dependent manner (6-11).

Active PAI-1 is quickly inactivated by a rapid, spontaneous conversion to the latent form (half-life 1-2 h) limiting its
therapeutic potential. Active PAI-1 has a reactive center loop (RCL) that contains a P1-P1′ site which extends from the body of the molecule. This site binds to and inactivates tPA. In latent PAI-1 this loop is retracted and becomes inserted between the A3 and A5 strands of the corresponding β-sheet, making the P1-P1′ site inaccessible to tPA (12,13). By restricting the movement of the A3 and A5 strands, as well as limiting the flexibility of the helix D region, it is possible to prevent insertion of the reactive loop between A3 and A5 and hence extend the half-life of PAI-1 (14).

Based on the known structure of active PAI-1, we identified amino acids that can be substituted with cysteine residues to produce disulfide bridges that constrain PAI-1 into a stable form that remains active for a prolonged period of time. We previously identified seven possible cysteine mutants (two- to six-point mutations, with an even number of cysteines) at the top and bottom portions of strands A3 and A5 and within the helix D region, or by a combination thereof, as potential candidate sites. The longest lasting half-life was observed for a β-sheet-top PAI-1 mutant (two-point mutations). We measured its activity for up to 700 h and determined that the half-life for this mutant could reach >6,900 h (14,15). In this study, we studied the ability of the VLHL PAI-1 mutant to inhibit tPA-mediated clot lysis in an in vitro model.

Materials and methods

Expression of VLHL PAI-1. The mutation of two amino acids (Gln197→Cys, Gly355→Cys) produced VLHL PAI-1 with a very long half-life >700 h. Construction of this protein was previously described (14). Briefly, the cDNA encoding PAI-1 was excised from the VLHL PAI-1 plasmid as a NdeI/Xho fragment. The PCR product of VLHL PAI-1 NdeI/Xho fragment was ligated into the pFastbac plasmid, which contains a 6His purification tag. To confirm mutations, the VLHL PAI-1 DNA construct was sequenced (MWG Biotech Inc., Mendenhall Oaks Parkway, NC).

Purification of VLHL PAI-1. VLHL PAI-1 was expressed and purified as described previously (16). Briefly, recombinant bacmid virus containing VLHL PAI-1 DNA was used to transfect Sf9 cells derived from Spodoptera Frugiperda (Fall Armyworm) using cellfectin reagent (Invitrogen) based on the manufacturer’s instructions. The virus was then used to infect Sf9 cells (~2x10^6/ml) on a large scale (1 liter of cell culture in a 2.8 liter Fernbach flask). The cells were harvested, lysed by two freeze-thaw cycles, and the lysate was centrifuged at 3,000 x g for 20 min to pellet cellular debris. The supernatant was transferred to a fresh tube for purification.

The supernatant was loaded onto a nickel resin-packed column (Invitrogen, Carlsbad, CA) using a peristaltic pump (GradiFrac system, Pharmacia Biotech) at a flow rate of 0.8 ml/min. The column was washed with buffer containing 40 mM imidazole in native buffer (50 mM NaH2PO4, 0.5 M NaCl, pH 8.0, containing protease inhibitors) at a flow rate of 1 ml/min until A280 reached baseline. The protein was then eluted using a gradient of 40-250 mM imidazole in native buffer at a flow rate of 1 ml/min. The peak fractions were dialyzed to remove imidazole, concentrated, and used for further analysis. Unused purified protein was stored at -80°C.

SDS-PAGE analysis. Electrophoresis of proteins was performed at room temperature using 4-12% SDS-polyacrylamide gradient gels under non-reducing conditions. Gels were stained with Colloidal Coomassie Blue (Invitrogen, Grand Island, NY).

Chromogenic assay of VLHL PAI-1 activity. The activity of PAI-1 was assessed by adding equal volumes of VLHL PAI-1; 50 μl diluted in H2O from a stock of 1.0 mg/ml in 20 mM HEPES, 250 mM imidazole, pH 8.0 to a concentration of 0.06 mg/ml and HMW uPA (American Diagnostica Inc., Stamford, CT) diluted from 1.0 mg/ml to 0.3 mg/ml in water. Following a 15-min incubation at room temperature, 50 μl of chromogenic uPA substrate (Spectrozyme® uPA, American Diagnostica Inc.), to a final concentration of 1.7 mM. Immediately following substrate addition, the absorbance at 405 nm was determined in a 96-well plate reader (VersaMax™ tunable microplate reader, Molecular Devices, Sunnyvale, CA) and was monitored every 20 sec for 50 min with uPA alone or with wPAI-1/uPA as controls.

Determination of time of fibrinolysis. Each well of a 96-well plate was filled with 100 μl of pooled normal human plasma, 20 μl of tPA (1.2 μg/ml), 40 μl tPA isotype factor (TF)/Ca2+ (ref. 824 from American Diagnostica Inc.; diluted 1:100), 20 μl 0.15 M NaCl or rFVIIa (from the same supplier and in the same buffer), and 20 μl of wPAI-1 or VLHL PAI-1 mutant in different concentrations (0.1, 1.0, 10.0 μg/ml). For inactivation studies, wPAI-1 and VLHL PAI-1 were incubated at 37°C overnight to induce conversion of wPAI-1 to the latent form. Clot formation and dissolution was monitored at 405 nm every 45 sec at 37°C using a SpectroMax Spectrophotometric plate reader (Molecular Devices). In this assay, clot formation is indicated by an increase in OD. Over time, the OD decreases due to clot dissolution by fibrinolytic processes, which reaches the initial OD when the clot has completely dissolved.

The areas under the fibrin aggregation curves were measured (Fig. 3). For the overall hemostatic potential determination (OHP⁰ψ₅), plasma samples were mixed with rFVIIa or tissue factor and tPA. For the overall coagulation potential measurements (OCPcontrol), plasma samples were mixed with rFVIIa or tissue factor as described above. For overall coagulation potential for PAI-1 (OCP¹ψ₅), tPA, rFVIIa or tissue factor and different concentration of wPAI-1 or VLHL PAI-1 were added. The overall clot protecting potential (OCP) or inhibition of clot lysis was calculated as: OCP = (OCP¹ψ₅ - OHP⁰ψ₅)/OCPcontrol. Nomenclature with some modifications are used as in ref. 17.

Results and discussion

VLHL PAI-1 characterization. As shown in Fig. 1, the single-step purification of VLHL PAI-1 yielded a ~95% pure protein (~10 mg from 1 liter of cell culture medium) as determined by densitometry of the SDS-PAGE gel bands.
The heavily stained ~40-kDa band represents active VLHL PAI-1, the faint ~45-kDa band is the latent form of VLHL PAI-1, and the weakly stained ~75-kDa band is a dimerized VLHL PAI-1. Identity of these protein bands was confirmed by liquid chromatography/mass spectrometry as previously reported (16,18).

Our initial VLHL PAI-1 construct contained the intein purification tag, which was expressed in E. coli. The yield of protein from this preparation was relatively low with only 1-2 mg from 1 liter of cell culture medium (15). A new vector containing a 6His tag was constructed and expressed in insect cells to increase the protein yield. As shown in Fig. 2 the half-life of the active form of the 6His tag VLHL PAI-1 construct was approximately the same as for the earlier construct (~700 h) (15). In all experiments, the activity of VLHL PAI-1 was normalized to VLHL PAI-1 protein concentrations.

Inhibition of fibrinolysis by VLHL PAI-1. Clot lysis by endogenous enzymes from pooled plasma takes ~10-12 h (19). To increase the speed of normal clot lysis and thus facilitate the analysis of the clot-protecting ability of VLHL PAI-1, tPA was added to pooled normal plasma prior to initiation of clot formation.

As indicated by the data displayed in Fig. 4, addition of wPAI-1 or VLHL PAI-1 increased the time of clot lysis. VLHL PAI-1 at 0.1 μg/ml was slightly less potent than wPAI-1. The lower activity of VLHL PAI-1 can be explained by Hagglof et al., who hypothesized that PAI-1s with Cys mutations in the A3 and A5 strands have reactive center loops that form a bulge on one side of the protein, which is shifted to the opposite side resulting in a more spherical molecule than active wPAI-1, where RCL is extended from the top of the protein molecule (20). This alteration in structure may lower the affinity of VLHL PAI-1 for tPA.

VLHL PAI-1 retained its clot-protecting abilities even with prolonged incubation. As shown in Fig. 4, overnight incubation (14 h) of wPAI-1 resulted in slight (10.0 μg/ml), significant (1.0 μg/ml) and complete (0.1 μg/ml) loss of clot protection abilities, while the clot-protective properties of VLHL PAI-1 remained relatively unchanged. Time to
complete lysis was ~5,500 sec for tPA alone and for tPA + 0.1 μg/ml wPAI-1, while it was unchanged for TPA + 0.1 μg/ml VLHL PAI-1 (~7,200 sec). Lysis velocity of VLHL PAI-1 (1.0 μg/ml, 10.0 μg/ml) remained unchanged. This indicates that VLHL PAI-1 was able to maintain an active form for a much longer period of time than wPAI-1.

In addition to its ability to block clot lysis induced by TF/Ca$^{2+}$, VLHL PAI-1 also blocked clot lysis initiated by rFVIIa (data not shown).

While the ability of PAI-1 to inhibit tPA-mediated fibrinolysis has long been known, the therapeutic potential of wPAI-1 has not been thoroughly investigated. This may be due in part to its rapid loss of protease inhibitory activity as a result of conversion to the latent form. Our findings suggest that VLHL PAI-1 may be a highly effective anti-fibrinolytic agent with therapeutic potential. The disulfide bridge of the recombinant VLHL PAI-1 prevents the reactive loop from embedding into the protein and thereby maintains the active conformation for a much longer period of time. VLHL PAI-1 was shown to be highly resistant to inactivation by heating at 37°C. The improved pharmacokinetics suggest that VLHL PAI-1 may be an ideal candidate for therapeutic use in various bleeding conditions including hemophilia, trauma, tooth extraction, surgical injury as well as in rebleeding following initial hemostasis (21). The ability of VLHL PAI-1 to inhibit fibrinolysis, and thus stabilize a clot may prove helpful when administered systemically to assist in stemming blood flow during episodes of severe bleeding. Administration of recombinant FVIIa has proved highly effective in treating uncontrolled bleeding in hemophilia. rFVIIa is also being investigated in clinical studies as a rapid procoagulant for controlling severe bleeding in trauma and surgery. In this study, we showed that VLHL PAI-1 inhibits fibrinolytic degradation of fibrin clots formed by rFVIIa in vitro. It is possible that administering the combination of the anti-fibrinolytic VLHL PAI-1 along with the procoagulant rFVIIa would be a more effective therapeutic treatment than rFVIIa alone for controlling severe bleeding conditions.

VLHL PAI-1 may also be useful for treating patients with defective PAI-1 proteins. Spontaneous bleeding is rarely observed in patients with PAI-1 mutations or deficiencies, whereas moderate hemorrhaging of the knees, elbows, nose and gums are usually triggered by mild trauma. In these patients, however, menstrual bleeding may be severe, and

Figure 4. Overnight incubation of VLHL PAI-1 does not decrease its ability to inhibit fibrinolysis. (A) wPAI-1 and VLHL PAI-1 without preincubation. (B) wPAI-1 and VLHL PAI-1 incubated at 37°C overnight prior to addition to plasma. Spectrophotometric tracing of clot formation and lysis (left column) for no tPA or PAI-1 added (green, g), tPA added without PAI-1 (black, b), or tPA added with various concentrations of wPAI-1 (blue: a, 10 ng/ml; b, 1.0 ng/ml; c, 0.1 ng/ml), or VLHL PAI-1 (red: d, 10 ng/ml; e, 1.0 ng/ml; f, 0.1 ng/ml). The overall clot protecting potential (OCPB) is expressed as percent (right column; color scheme as above).
prolonged bleeding after surgery is common (22-24). VLHL PAI-1 may be used for treating these patients under these conditions. Another potential application is the protective role of VLHL PAI-1 in reducing early mortality rate in lethal pneumococcal pneumonia. It has been found by Lim et al that administration of exogenous PAI-1 prevented lung injury and reduced mortality rate during severe pneumococcal pneumonia in vivo (25).

In conclusion, these studies demonstrate that VLHL PAI-1 effectively protects fibrin clots from degradation by the fibrinolytic system. VLHL PAI-1 with its very long half-life offers possible therapeutic applications for surgery and trauma patients and for other patients where premature clot lysis may be dangerous to the individual.

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