



Systemic or topical application of plasminogen activator inhibitor with extended half-life (VLHL PAI-1) reduces bleeding time and total blood loss

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Received April 12, 2010; Accepted May 26, 2010

DOI: 10.3892/ijmm_00000491

Abstract. Civilian and military trauma patients consist of a disproportional number of young people, causing a considerable burden to society in terms of disability and premature death. Hemorrhage is a leading cause of mortality in this group of patients and the novel methods to reduce bleeding would be welcomed. Management of bleeding following major trauma includes hemostatic agents that offer effective clotting. However a very limited number of agents control secondary bleeding triggered by lysis of the clot. Fibrinolysis depends on the balance between tissue plasminogen activator (tPA), activating plasminogen to plasmin initiating fibrinolysis, and plasminogen activator inhibitor type 1 (PAI-1) inhibiting tPA and preventing lysis. The drugs available on the market that prevent the activation of plasminogen have been used successfully, but have some side effects and limited efficacy for the control of localized bleeding in the surgical setting. Inhibitors of tPA, initiator of clot fibrinolysis, have not yet found their way into the clinical arena. Plasminogen activator inhibitor-1, the major specific inhibitor of tPA, can be used to limit fibrinolysis. Unfortunately, PAI-1 has a short half-life of ~2 h and is rapidly converted to the latent form. A recombinant PAI-1 with very long half-life developed in our laboratory (a two-point mutant, VLHL PAI-1, half-life over 700 h) has clinical potential as an agent to promote hemostasis in several scenarios including surgical injury, trauma, and PAI-1 deficiency. Here we report testing of VLHL PAI-1 as a potent inactivator of fibrinolysis reducing total blood loss while applied systemically or topically in experimental animals. The very long half-life of VLHL PAI-1 may provide an advantage in the important physiological mechanism to protect clots from premature dissolution, when applied

topically or systemically to prevent excessive bleeding in the surgical and trauma setting and possibly in PAI-1 deficient patients.

Introduction

The recovery of hemorrhagic trauma patients has improved over recent years but there is still a need to control bleeding in order to reduce morbidity and mortality (1,2). It has been reported that regardless of advances in trauma care, uncontrolled bleeding contributes up to 40% of trauma patient's deaths (3). Civilian and military trauma cases affect a disproportional number of young people, thus the burden to society in terms of disability and premature death, is considerable.

Management of bleeding following major trauma includes hemostatic agents that offer effective clotting but a very limited number of agents are available to control secondary bleeding set off by fibrinolysis. Fibrinolysis depends on the balance between tissue plasminogen activator (tPA), activating plasminogen to plasmin causing fibrinolysis, and plasminogen activator inhibitor type 1 (PAI-1) inhibiting tPA and thus preventing fibrinolysis. There are drugs on the market that prevent the activation of plasminogen through direct interaction with the lysine binding site of plasminogen. The inhibition of plasmin activity has been explored by using 6-amino caproic acid (ϵ -amino caproic acid, Amicar[®], Lederle) or tranexamic acid. However, while these drugs have been used successfully, they have some side effects. In addition they have limited efficacy for the control of localized bleeding in the surgical setting (4).

Inhibitors of tPA, initiator of clot fibrinolysis, have not yet found their way into the clinical arena. The plasminogen activator inhibitor-1 is the major specific inhibitor of tPA and can be used to limit fibrinolysis. The major clinical obstacle is its short half-life of ~2 h of PAI-1. The wild-type PAI-1 converts from active to latent form. A form of PAI-1 with very long half-life, which has been developed in our laboratory (a two-point mutant, VLHL PAI-1, half-life over 700 h) has clinical potential as an agent to promote hemostasis in several scenarios including surgical injury, trauma, and PAI-1 deficiency, as well as in rebleeding following initial hemostasis. In our previous publications we have reported that VLHL PAI-1

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Key words: PAI-1, blood clotting, bleeding time, blood loss

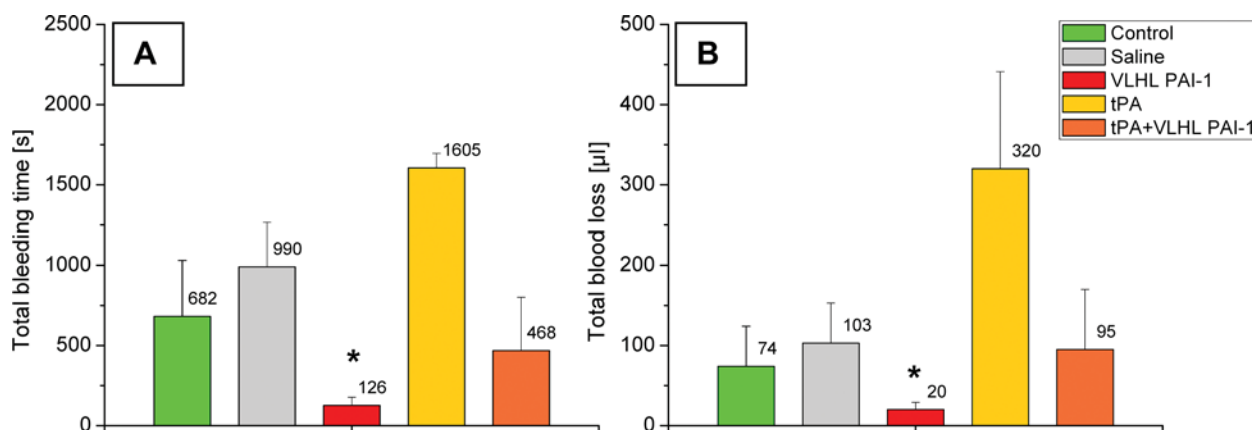


Figure 1. Total time of bleeding (A) and total blood loss (B) in control animals and animals infused with VLHL PAI-1 and VLHL PAI-1+tPA. $n=4$, asterisk indicates statistical significance at a $P<0.05$ between VLHL PAI-1 group and controls.

is an effective inactivator of fibrinolysis *ex vivo* and *in vivo* in an animal model while administered systemically (5-7). The current study provides evidence that VLHL PAI-1 is equally potent as an inactivator of fibrinolysis and total blood loss when applied either systemically or topically in experimental animals.

Materials and methods

Proteins. The mutation of two amino acids (Gln197→Cys, Gly355→Cys) in human wPAI-1 [SwissPROT P05121 (8)] produces VLHL PAI-1 with half-life of over 700 h (9). Composition of this protein and purification were described previously by our group (5,10). Fully active human tPA, product number HTPA-TC was purchased from Molecular Innovations, Novi, MI.

Animals. Mice used were retired male breeders, C57BL/6J, Jackson Labs, Bar Harbor, Maine, fed *ad libitum*. All animal experiments were performed according to the institutional ethics guidelines.

Systemic administration. Animals were anesthetized via gas inhalation (1.0-2.0% isoflurane). The left jugular vein was surgically exposed and cannulated with a PE 10 catheter to administer saline, tPA and PAI-1. Flow rate was controlled with a syringe pump (Medex, Inc., Duluth, GA). All animals received the same volume of fluids. The pump rate used was 0.3 ml/h for a total volume of 300 μ l. The tail was immersed in warm (37°C) saline during the infusion time. Immediately after the infusion, 5 mm of the tip of the tail was cut off using a fresh scalpel blade and placed in a vial of warmed (37°C) saline (50 ml). Bleeding episodes were monitored and recorded for 30 min. Animals were euthanized at the end of the bleeding observation period. Animals were divided into 5 groups ($n=4$).

Animals in group I served as a control group that received no surgery or systemic infusion, but had their tails warmed for 1 h prior to clipping. Animals in group II received surgery and saline infusion for 1 h prior to clipping their tails. Animals in group III were infused with saline for 30 min followed by VLHL PAI-1 (total dose 225 μ g) for 30 min. Animals in

group IV were infused with tPA (total dose 108 μ g) for 30 min followed by saline for 30 min prior to clipping the tail. Animals in group V were infused with tPA (total dose 108 μ g) for 30 min followed by VLHL PAI-1 (total dose 225 μ g) for 30 min prior to clipping the tail.

External application. Animals were anesthetized via gas inhalation (1.0-2.0% isoflurane). Five millimetres of the tip of the tail was cut off using a fresh scalpel blade and placed into a 1.5 ml vial containing either 1.0 ml of saline or 1.0 ml of VLHL PAI-1. Bleeding episodes were monitored and recorded for 30 min. Animals were euthanized at the end of the bleeding observation period.

Animals were divided into 4 groups of 8 animals each. In group Ia (control) the animal's clipped tails were placed in saline. In group IIa tails were placed in 300 μ g VLHL PAI-1/ml, in group IIIa, in 75 μ g VLHL PAI-1/ml, and in group IVa in 19 μ g VLHL PAI-1/ml.

All bleeding episodes were timed and a summary yielded a total bleeding time. Hemoglobin concentration was determined in all collection vials spectrophotometrically and converted to blood loss volume utilizing a standardized curve.

Statistical analysis was carried out using Origin 8 program (OriginLab Corporation, Northampton, MA 01060). Statistical significance was set at a $P<0.05$ as calculated using ANOVA test.

Results

Surgery and injection of saline slightly increase bleeding time and blood loss but it was not statistically significant. The positive control group that received infusion of tPA showed an increase time of bleeding and blood loss. Infusion of tPA followed by infusion of VLHL PAI-1 brings bleeding time and blood loss to the control values. However, statistically significant reduction of bleeding time and total blood loss was seen when only VLHL PAI-1 was infused (Fig. 1).

As can be seen in Fig. 2 when animals were treated topically with VLHL PAI-1 differences in total bleeding time between treated and non-treated animals were smaller and

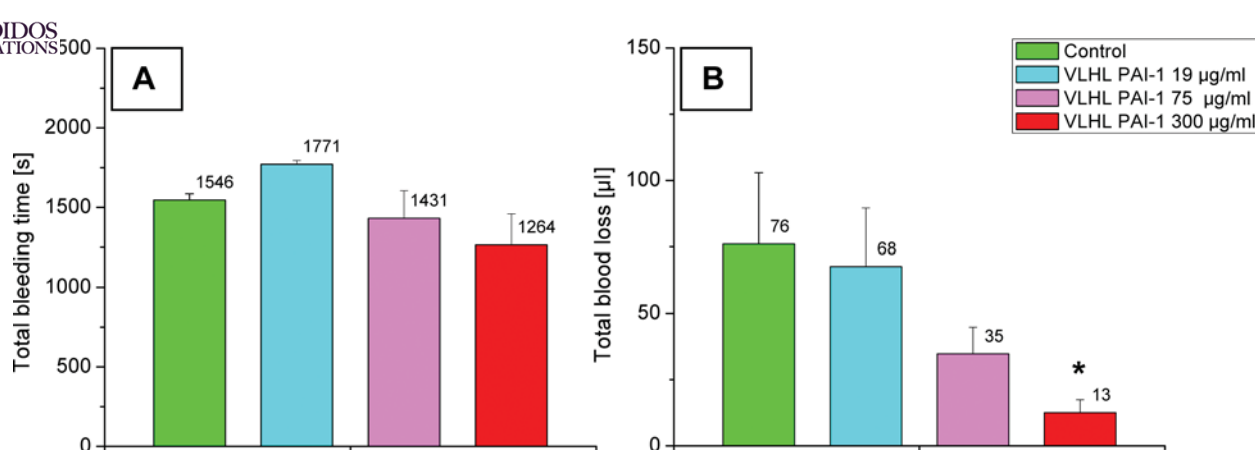


Figure 2. Total time of bleeding (A) and total blood loss (B) in control animals and animals treated topically with VLHL PAI-1 at different concentrations. n=8, asterisk indicates statistical significance at a $P<0.05$ between VLHL PAI-1 group and control.

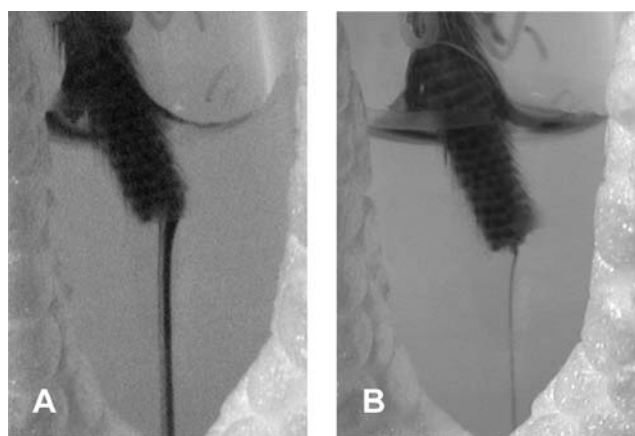


Figure 3. Bleeding from multiple or single blood vessels after amputation of tail tip immersed in saline or saline solution of VLHL PAI-1. Bleeding time was recorded when bleeding intensity was high (A) or low (B). The more reliable measure of reducing bleeding by VLHL PAI-1 is total blood loss.

not significant. We have noticed in this group that animals bled frequently (on/off) but the intensity of bleeding was drastically different as can be seen in Fig. 3. Only the bleeding episodes were recorded and not the intensity of the blood stream. Bleeding from multiple or single vessels were recorded equally. We conclude that at least in these groups total time of bleeding was a weak indicator of blood loss.

Remarkably, as can be seen in Figs. 1 and 2 animals treated topically and systemically with similar dose of VLHL PAI-1 showed practically the same reduction of blood loss. Control groups, whether treated topically or systemically were also the same. Furthermore, when applied topically, reduction of total blood loss was VLHL PAI-1 concentration dependent with 50% and 80% blood loss reduction at 75 µg/ml and 300 µg/ml respectively.

Discussion

Previously we have reported that systemic application of VLHL PAI-1 reduces bleeding time and total blood loss in

C57BL/6J mouse (7). In this study we have used a larger number of animals and added an additional control group. This group included animals in whom no surgery was done and there was no treatment.

VLHL PAI-1 is the specific inhibitor of tPA and acts by forming a 1:1 complex, followed by the formation of a covalent bond between the active site of the protease and the reactive center of the serpin. PAI-1 binds to fibrin but not to fibrinogen and thus can localize itself at the sites of injury (11). Plasminogen activator inhibitor-1 accumulated within thrombi retains its complete tissue-type plasminogen inhibitory activity protecting the clot from premature dissolution (12). All these properties make PAI-1 an attractive anti fibrinolytic agent.

Usage of inhibitors of fibrinolysis to limit bleeding was explored before by others. Reilly *et al* found that fibrin clot lysis in rats was inhibited in a dose-responsive manner by infusion of active wild-type of PAI-1 (13). In other *in vivo* studies using a rabbit ear model of blood loss it was found that the active form of wPAI-1 and ϵ -amino caproic acid (EACA) reduce the bleeding induced by tPA. However, wPAI-1 reduced hemorrhage more effectively (14). These results support our findings and indicate that PAI-1 inhibits fibrinolysis *in vivo*. Nevertheless, one of the most critical hurdles in using wPAI-1 as a hemostatic drug is its short half-life ($t_{1/2}=2$ h) (15,16). VLHL PAI-1 with its very long half-life can eliminate this problem.

In conclusion, we wanted to evaluate the effect of VLHL PAI-1 on hemostasis and we have demonstrated that recombinant human VLHL PAI-1 administered topically or systemically is an efficient inhibitor of fibrin clot degradation. It reduces total blood loss and total time of bleeding. However, our results show that in some cases time of bleeding alone may be a weak indicator of blood clot protecting activity of the hemostatic agents. Very long half-life PAI-1 when applied topically or systemically may provide an important physiological mechanism to protect clots from premature dissolution in the surgical and trauma setting and might provide a promising remedy in PAI-1 deficiency.

Acknowledgements

We thank Dr R. Hart (President, PharmaIP LLC, Greenwich, CT, USA) for helpful remarks, discussions and support. This study was supported in part by grants from: PharmaIP LLC, and the Frank D. Stranahan Endowment Fund for Oncological Research.

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