



Identification and Characterization of Plasmin-Independent Thrombolytic Enzymes

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RATIONALE: Current thrombolytic agents activate plasminogen to plasmin which triggers fibrinolysis to dissolve thrombi. Since plasmin is a nonspecific proteolytic enzyme, all of the current plasmin-dependent thrombolytics lead to serious hemorrhagic complications, demanding a new class of fibrinolytic enzymes independent from plasmin activation and undesirable side effects. We speculated that the mammalian version of bacterial heat-shock proteins could selectively degrade intravascular thrombi, a typical example of a highly aggregated protein mixture.

OBJECTIVE: The objective of this study is to identify enzymes that can dissolve intravascular thrombi specifically without affecting fibrinogen and fibronectin so that the wound healing processes remain uninterrupted and tissues are not damaged. In this study, HtrA (high-temperature requirement A) proteins were tested for its specific proteolytic activity on intravascular thrombi independently from plasmin activation.

METHODS AND RESULTS: HtrA1 and HtrA2/Omi proteins, collectively called as HtrAs, lysed ex vivo blood thrombi by degrading fibrin polymers. The thrombolysis by HtrAs was plasmin-independent and specific to vascular thrombi without causing the systemic activation of plasminogen and preventing nonspecific proteolysis of other proteins including fibrinogen and fibronectin. As expected, HtrAs did not disturb clotting and wound healing of excised wounds from mouse skin. It was further confirmed in a tail bleeding and a rebleeding assay that HtrAs allowed normal clotting and maintenance of clot stability in wounds, unlike other thrombolytics. Most importantly, HtrAs completely dissolved blood thrombi in tail thrombosis mice, and the intravenous injection of HtrAs to mice with pulmonary embolism completely dissolved intravascular thrombi and thus rescued thromboembolism.

CONCLUSIONS: Here, we identified HtrA1 and HtrA2/Omi as plasmin-independent and highly specific thrombolytics that can dissolve intravascular thrombi specifically without bleeding risk. This work is the first report of a plasmin-independent thrombolytic pathway, providing HtrA1 and HtrA2/Omi as ideal therapeutic candidates for various thrombotic diseases without hemorrhagic complications.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: fibrinolysis ■ myocardial infarction ■ plasminogen ■ proteolysis ■ thrombosis

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Thrombi are blood clots that form constantly by injuries or unknown physiological reasons in a human body.¹⁻³ Although the formation of a thrombus at the site of injury is essential for wound healing, an intravascular thrombus resulting in occlusive blood clots do not have

such benefits. Unlike its counterpart, the occlusive intravascular thrombus obstructs the flow of blood through the circulatory system, causing fatal vascular diseases, such as ischemic stroke, acute myocardial infarction, pulmonary embolism, venous thromboembolism, etc.⁴⁻⁶

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Novelty and Significance

What Is Known?

- Plasmin is the key enzyme in the fibrinolytic cascade dissolving blood clots.
- Plasmin functions as a nonspecific protease that trigger degradation of a variety of proteins in plasma and tissues as well as destroying blood clots.
- Current thrombolytic enzymes are all plasmin-dependent and their therapeutic use may trigger hemorrhage and tissue injury complications.

What New Information Does This Article Contribute?

- HtrAs (high-temperature requirement As) specifically recognize and degrade intravascular thrombi without activation of plasminogen.
- HtrAs do not have nonspecific proteolytic activities so that the enzymes dissolve only intravascular thrombi without affecting other blood proteins.
- Unlike plasmin and its activators, HtrAs do not interfere with the wound healing.
- The proteolytic function of bacterial HtrA towards damaged or misfolded proteins seems to be evolutionary conserved in mammals as HtrA1 and HtrA2 degrade blood thrombi which are a complicated mixture of heavily damaged and misfolded proteins.

Thrombosis is a common underlying pathology for many cardiovascular diseases. Because of its serious consequences, there has been decades of efforts to develop thrombolytic agents. Current thrombolytic agents such as streptokinase, urokinase, recombinant tPA (tissue-type plasminogen activator), etc, activate the fibrinolytic system by converting an inactive zymogen, plasminogen, to a proteolytically active plasmin to initiate lysis of the thrombus. However, the nonspecific nature of plasmin results in serious complications related to tissue damage and bleeding, which limits the use of plasminogen activators in patients. Here, we report that HtrAs (HtrA1 and HtrA2/Omi) degraded the cross-linked fibrin mesh of the intravascular thrombus without activation of plasminogen. HtrAs worked independently from the wound healing processes, unlike plasmin. In animal models, intravenous injection of HtrAs dissolved intravascular thrombi and thereby completely rescued the death of the mice from pulmonary thromboembolism without any side effect or toxicity. This work demonstrated that HtrAs could be developed as a specific thrombolytics for thrombosis.

Nonstandard Abbreviations and Acronyms

FN	fibronectin
HtrA	high-temperature requirement A
SK	streptokinase
tPA	tissue-type plasminogen activator
UK	urokinase

Thrombi at a wound site are dissolved by plasmin, a trypsin-like protease involved in various physiological processes, such as inflammation, embryo development, and cancer progression.^{7,8} The dissolution of intravascular thrombi also depends on plasmin by converting inactive zymogen plasminogen to active proteolytic plasmin using plasminogen activators, such as SK (streptokinase), UK (urokinase), and tPA (tissue-type plasminogen activator).^{4,7} Unfortunately, the plasmin generated during thrombolytic therapies with plasminogen activators contributes to fatal bleeding and tissue damage.^{9–11} This phenomenon arises from the nonspecific proteolytic activity of plasmin which degrades not only the fibrin clots but also the various proteins involved in tissue repair and wound healing processes.^{12,13} In addition to them, plasmin also degrades fibrinogen, an essential protein for

platelet aggregation and clot stability, interfering with the coagulation of blood during wound healing processes.¹⁴ Thus, the bleeding complication and severe tissue damage associated with plasmin-dependent thrombolytic therapy have limited its application to the patients with high bleeding risk,^{9,10} demanding for a new class of fibrinolytic enzymes independent from plasmin activation and serious side effects.

Except for injury many genetic and acquired risk factors including age, surgery, obesity, cancer, and smoking might contribute to generate intravascular thrombi. To avoid fatal obstructions caused by intravascular thrombi, it seems logical that a plasmin-independent, yet unidentified fibrinolytic enzyme for dissolution of intravascular thrombi would be present in blood.^{15–17} Identification of a new class of fibrinolytic enzyme independent from plasmin activation pathway would contribute not only to a scientific understanding of human physiology but also to the development of a thrombolytic agent free from any unwanted side effects.

A thrombus is a complicated mixture of heavily damaged proteins.^{18–20} In bacteria, denatured proteins are repaired by heat-shock proteins, whereas more seriously damaged proteins are degraded by HtrA (high-temperature requirement A) protein in stress condition for survival.^{21–23} Both the amino acid sequence and the

3-dimensional structures of HtrA are evolutionary well-conserved^{24–26} with genetic divergence into 4 genes in mammals.^{26,27} Because the bacterial heat-shock proteins are functionally conserved as chaperones in higher animals including humans,²⁸ we speculated that the mammalian HtrAs could function as a thrombolytic enzyme for the degradation of intravascular thrombi, a typical example of a highly damaged and aggregated protein mixture in blood. In this study, the mammalian versions of bacterial HtrAs were tested for its thrombolytic activity and HtrA1 and HtrA2/Omi were identified as highly specific thrombolytic enzymes for specific degradation of intravascular thrombi without the activation of plasmin, preserving the wound healing processes undisturbed.

METHODS

The data that support the findings of this study are available from the corresponding authors upon reasonable request. Detailed methods are available in the [Data Supplement](#).

RESULTS

HtrAs Lysed Blood Thrombi by Degrading Fibrin Polymers

Bacterial HtrA diverged into 4 mammalian HtrA proteins, HtrA1 to HtrA4, in higher animals. Recombinant HtrA1 and HtrA2/Omi, collectively called as HtrAs in this study, were prepared (Figure I in the [Data Supplement](#)) and treated to the ex vivo thrombi for thrombolytic activity along with HtrA3 and HtrA4 in the presence of fresh plasma. Among these, HtrA1 and HtrA2/Omi showed significant thrombolytic activities unlike HtrA3 and HtrA4 (Figure II in the [Data Supplement](#)) and were selected to treat the ex vivo thrombi for thrombolytic activity in the presence of fresh plasma with other thrombolytic agents (Figure 1A and 1B). The blood clot lysis measured by the weight of thrombus was 72% of the control in HtrA1, 48% in plasmin, 38% in SK, and 48% in UK, respectively (Figure 1A), whereas it was 71% of the control in HtrA2/Omi, 48% in plasmin, 40% in SK, and 49% in tPA, respectively (Figure 1B).

Thrombus degradation by HtrAs was also investigated by the quantification of fibrin degradation products in the thrombolysis solution (Figure 1C and 1D). Consistent with the greatest magnitude of thrombolysis by HtrA treatment, the levels of fibrin degradation products, D-dimer and fibrin degradation products, by HtrA1 (Figure 1C) and HtrA2/Omi (Figure 1D) were the highest among the cotested thrombolytics. SDS-PAGE followed by subsequent Q-TOF (quadrupole time-of-flight mass spectrometer) analysis of the enzyme-treated thrombus solution also showed a series of proteolyzed FN (fibronectin) and fibrin gamma chain peptide fragments of cross-linked fibrin mesh, confirming the degradation

of fibrin polymer of thrombus by HtrAs (Figure III in the [Data Supplement](#)). In addition to the fibrin degradation, HtrA treatments dissolved platelet aggregates most effectively among the tested thrombolytic agents (Figure 1E through 1H). Compared with the control, HtrA treatments resulted in complete disappearance of platelet aggregates, whereas plasmin, UK, and tPA showed moderate dissolution of aggregates. Unlike the platelet aggregates, HtrAs did not affect free platelets (Figure IV in the [Data Supplement](#)). Taken together, HtrA1 and HtrA2/Omi demonstrated a distinct thrombolytic activity through fibrin degradation in vitro.

Independent From Plasminogen Activation, HtrAs Lysed Thrombi Upon Regulation by Plasma

The unique thrombolytic characteristic of HtrAs led us to investigate the effect of HtrAs in the blood clotting and wound healing. The amidolytic assay using plasmin-specific fluorogenic substrates showed that HtrAs did not activate plasminogen, whereas cotested current thrombolytic agents did (Figure 2A and 2B, left). SDS-PAGE analysis also confirmed that HtrAs did not activate plasminogen to plasmin unlike other thrombolytics (Figure 2A and 2B, right). These results indicated that the thrombolysis by HtrAs does not involve plasmin, a key enzyme of the fibrinolysis in wound healing processes. The plasmin-independent thrombolytic property of HtrAs led us to identify and characterize the cleavage sites of HtrAs in cross-linked fibrin mesh. The Edman sequencing analysis of HtrA-digested fibrin clots clearly showed that HtrA1 and HtrA2/Omi have distinct cutting sites in α , β , and γ chains of fibrin (Figure 2C and 2D). After identifying the thrombolytic activities and cleavage sites of HtrAs, we investigated the enzymatic kinetics of HtrAs in the presence of citrated blood plasma. The Lineweaver-Burk plot showed that blood plasma functions as an uncompetitive inhibitor for plasmin and a competitive inhibitor for SK and UK while acting as a noncompetitive inhibitor for HtrAs on thrombi (Figure 2E and 2F; and Table I in the [Data Supplement](#)). The noncompetitive inhibition of HtrAs by plasma suggests the possible presence of unknown inhibitor(s) in plasma for the regulatory site of HtrAs to modulate proteolytic efficiency.

HtrAs Lysed Fibrin Clots Without Causing Nonspecific Proteolysis

Because the thrombolytic activities of HtrAs were independent of plasminogen activation, we further investigated whether HtrAs degrade fibrin clots specifically instead of nonspecific proteolysis. Fibrin clots were prepared by incubating fibrinogen with thrombin followed

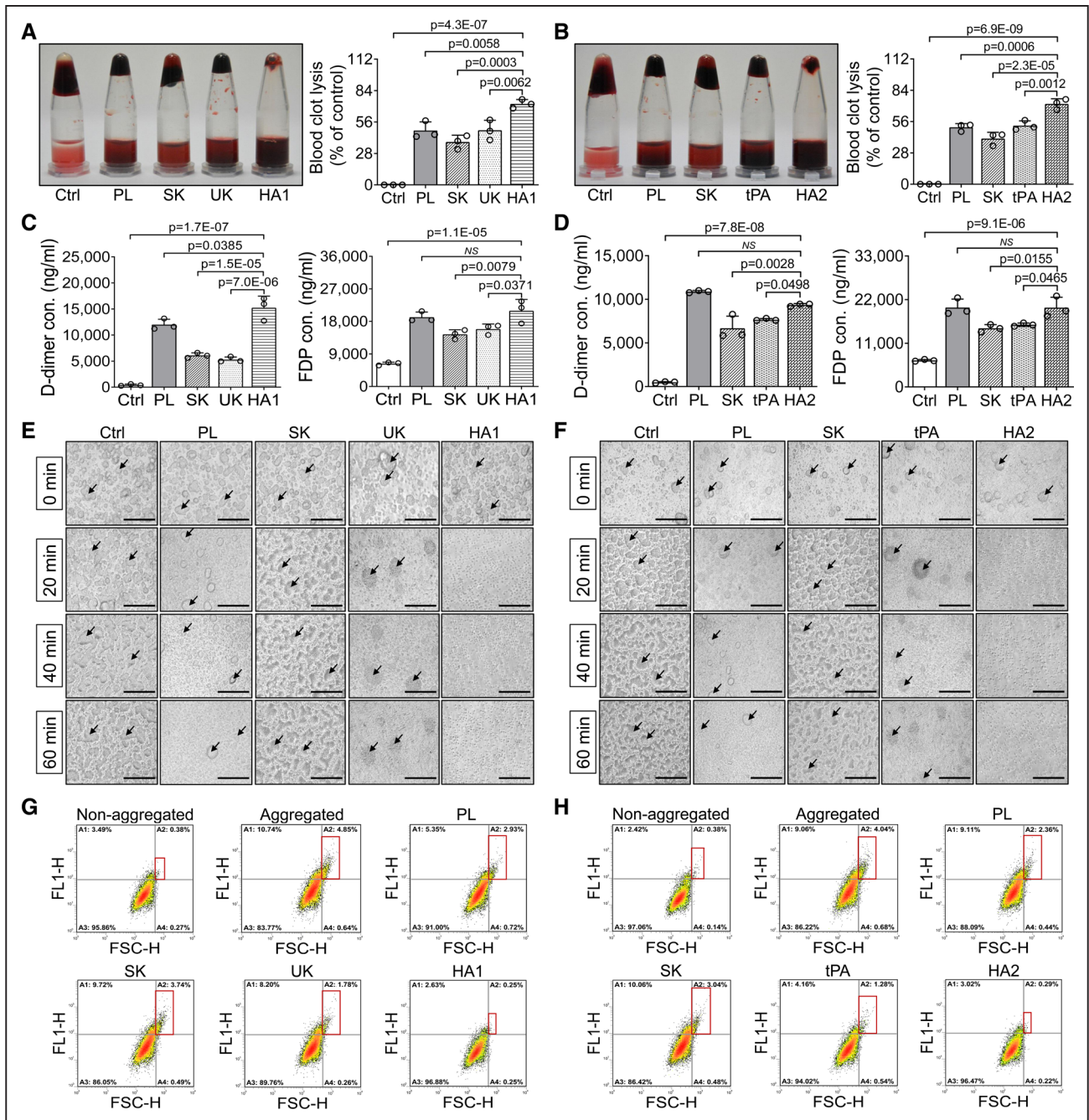


Figure 1. The ex vivo thrombolytic effect of HtrAs (high-temperature requirement As).

A and B, Effect of thrombolytics on blood thrombus. The thrombolytic activity was measured by the weights of solid blood clots after treatment with the thrombolytics as indicated (n=3). **C and D**, Effect of thrombolytics on fibrin degradation. Fibrinolysis was measured by quantitating the amount of fibrin degradation products (FDPs) and D-dimers in the lysed solution using ELISA after treatments with thrombolytics as indicated (n=3). **E and F**, Effect of thrombolytics on platelet aggregates. Platelet aggregates (black arrows) were prepared by ADP followed by treatment with thrombolytics as indicated. The antiplatelet activity of platelet aggregation was determined by the disappearances of platelet aggregates under the Nikon TE2000-S microscope. Scale bar: 200 μm. **G and H**, The antiplatelet aggregation activities of HtrAs were carried out with washed human platelets. Dot plot depicting the platelets labeled with carboxyfluorescein succinimidyl ester was measured by flow cytometry after enzyme treatment. Aggregated platelets are enclosed with the red box in quadrant A2 analyzed by comparing the dots of aggregated platelets in percentage. All populations are plotted against FSC-H and FL1-H. Error bars, mean±SD. Data (**A–D**) were analyzed using one-way ANOVA with Tukey multiple comparison test based on the normality of data assessed by the Shapiro-Wilk normality test. Ctrl indicates control; Con, concentration; FSC-H, forward scatter height; FL1-H, fluorescence intensity (height) in the flow cytometry channel 1; HA1, HtrA1; HA2, HtrA2/Omi; PL, plasmin; SK, streptokinase; tPA, tissue-type plasminogen activator; and UK: urokinase.

by the addition of the thrombolytics in the presence of citrated fresh human plasma. The fibrin clot degradation assay showed that HtrAs most efficiently degraded

thrombin converted fibrin clots among the tested thrombolytics in the presence of plasma (Figure 3A and 3B),

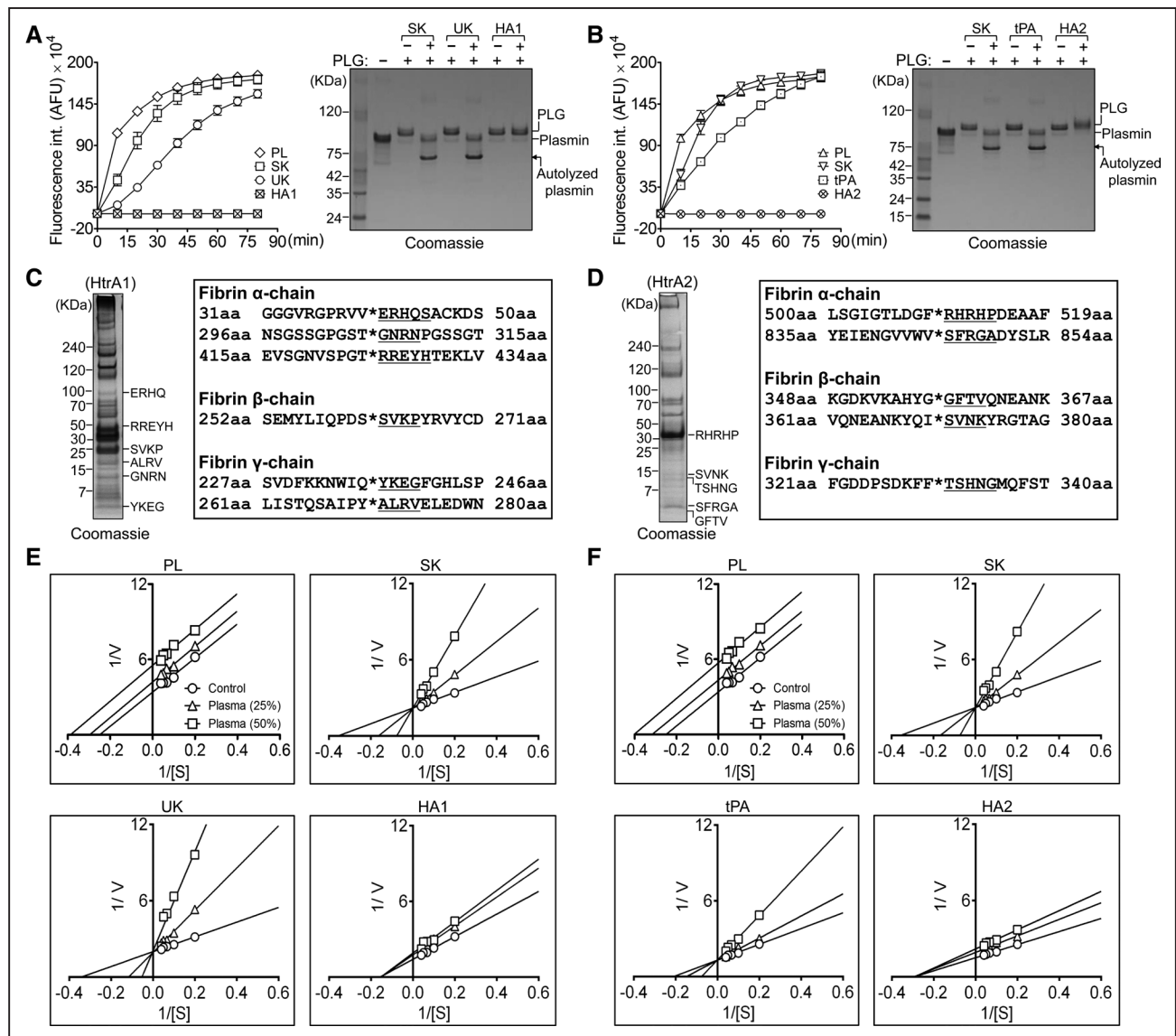


Figure 2. The plasmin-independent thrombolytic activities of HtrAs (high-temperature requirement As) upon regulation by plasma. **A** and **B**, left, Analysis of plasminogen activation by the amidolytic assay. Plasminogen was incubated with the indicated thrombolytics in the presence of plasmin-specific fluorogenic substrate Boc-Glu-Lys-Lys-MCA. The activation of plasminogen to plasmin was monitored by measuring fluorescence intensities from lysed substrates by plasmin. **A** and **B**, right, Analysis of plasminogen activation by Coomassie blue-stained SDS-PAGE. **C** and **D**, The cleavage sites of HA1 (HtrA1) and HA2 (HtrA2/Omi) enzymes in cross-linked fibrin α , β , and γ chains were determined by N-terminal Edman sequencing analysis. The cutting sites in amino acid sequences of fibrin chains are shown with star marks (*). **E** and **F**, The enzyme kinetics of the thrombolytics in the presence of blood plasma. Platelet-rich plasma clots were incubated with indicated thrombolytics 2 mg/mL with 50 mmol/L Tris-HCl (circle), Tris-HCl with 25% (v/v) fresh human plasma (triangle), or Tris-HCl with 50% (v/v) fresh human plasma (square). V_{max} and K_m were calculated from the OD_{415} values of the reaction using a Lineweaver-Burk plot equation. AFU indicates arbitrary fluorescence unit; PLG, plasminogen; PL, plasmin; [S], concentration of substrate; SK, streptokinase; tPA, tissue-type plasminogen activator; UK, urokinase; and V, velocity.

implying the possibility of fibrinolysis by HtrAs in the blood.

Next, we investigated the effect of HtrAs on native fibrinogens and FNs, the mediators of wound healing, a vital process disturbed by current thrombolytic agents. Coomassie staining revealed that HtrAs did not degrade fibrinogen in the presence of plasma, whereas other thrombolytics did (Figure 3C and 3D). Similarly, HtrAs did not degrade FNs from plasma and cellular matrix in the presence of plasma, whereas all the other thrombolytics

showed evident degradation (Figure 3E and 3F). FN is known to bind to the polymerized fibrin to form a blood clot in a wound site,²⁹ which means that intact FN is involved in wound healing processes. In addition, all tested thrombolytics, except HtrAs, degraded the native circulatory fibrinogen and FN (Figure 3C through 3F). It suggested that HtrAs might not disturb wound healing through the proteolysis of circulatory fibrinogen and FN, unlike cotested thrombolytics.

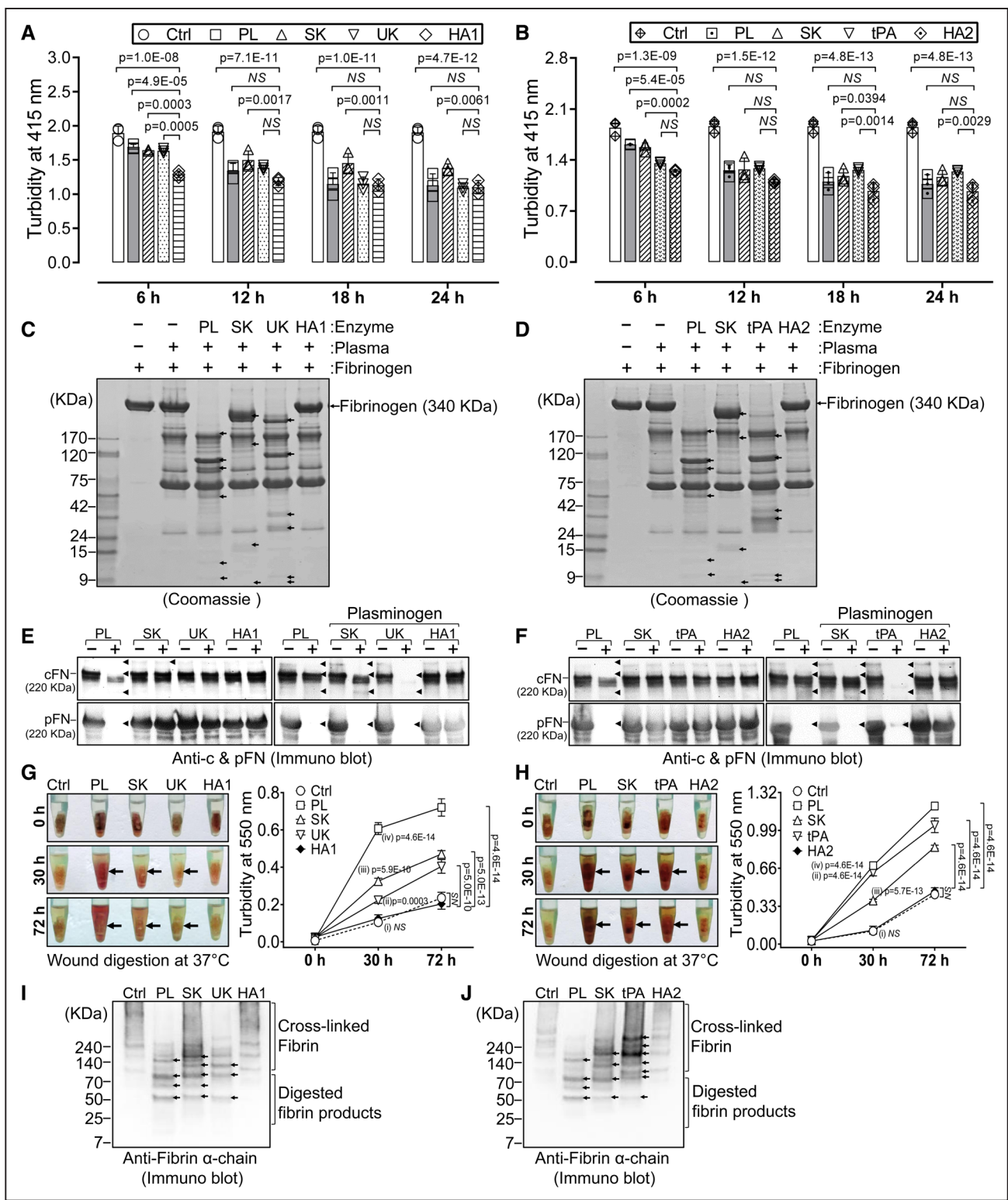


Figure 3. The selective proteolytic activities of HtrAs (high-temperature requirement As) on fibrin clots. **A** and **B**, Fibrinolytic activity of HA1 (HtrA1) and HA2 (HtrA2/Omi) in the presence of blood plasma. Fibrin clots prepared by incubation of FGN (fibrinogen) with thrombin were incubated at 37°C for 24 h with either 50 mmol/L Tris-HCl as control (Ctrl) or thrombolytics (n=3). The lysis of fibrin clots was measured at 415 nm wavelength using a spectrophotometer. **C** and **D**, Proteolytic activity of the thrombolytics on FGN in the presence of plasma. Aliquots of FGN were incubated with either 50 mmol/L Tris-HCl as Ctrl or thrombolytics at 37°C for 3 h. The degradation of FGN was observed by SDS-PAGE stained with Coomassie blue. **E** and **F**, Proteolytic effect of HA1 and HA2 on purified cFN (cellular fibronectin) and pFN (plasma fibronectin) in the presence of blood plasma. Cellular or pFN was incubated with indicated thrombolytics in the absence or presence of plasminogen at 37°C for 3 h. The reaction samples were separated on 4%–12% SDS gel followed by immunostaining with anti-cFN (anti-cellular) or anti-pFN (plasma fibronectin) antibodies. **G** and **H**, Proteolytic activity of HA1 and HA2 on wounds in plasma presence. (Continued)

The actual effects of HtrAs on wound thrombi were confirmed with mice tail skin (Figure 3G and 3H). HtrAs successfully demonstrated no significant wound digestion, unlike cotested enzymes. Furthermore, the Western blotting analysis of digested wound samples using fibrin specific antibody showed no digestion of cross-linked fibrin in the HtrA treatments unlike the treatments with plasmin, SK, UK, or tPA (Figure 3I and 3J). This observation implies that HtrAs selectively dissolved intravascular thrombi, but not wound thrombi, preserving the wound healing process without risking survival.

The classical platelet marker CD41, fibrinogen receptor integrin alpha IIb, is known to bind to fibrinogen, FN, vitronectin, and von Willebrand factor, and the CD41/CD61 complex is required for platelet activation, aggregation, and clotting.^{30,31} To investigate the mechanism for antiplatelet aggregation properties of HtrAs, the aggregated platelet cell lysate was treated with thrombolytics (2 mg/mL) and observed by Western blotting using anti-CD41. HtrAs were shown to modify CD41, distinctly from the modifications by plasmin and tPA, through proteolytic activities, whereas SK and UK failed to show any proteolysis (Figure V in the [Data Supplement](#)). Taken together, our results suggest that HtrAs precisely lyse blood thrombi without the systemic activation of plasminogen, preventing tissue damages and defects in wound healing.

We further investigated the specificity of proteolytic activities of HtrAs using the mouse and human tissues (Figure 4). Although the treatments of other thrombolytics resulted in nonspecific proteolytic activities toward various proteins from mouse and human, HtrAs showed greatly reduced proteolytic activities instead of widespread general proteolysis in tissues as well as platelets from mouse and human. These nonspecific proteolytic activities of current thrombolytics well explain their serious side effects of tissue damages and bleeding. In contrast, the specificity of HtrAs seems to assure improved safety without any side effects from nonspecific proteolytic activity.

Thrombolytic HtrAs Did Not Disturb Wound Healing Processes in Mice

A tail bleeding and the rebleeding assay was conducted to investigate whether HtrAs affect wound healing, a crucial process for survival (Figure 5). For clotting assay in the tail bleeding experiment, tails were amputated from anesthetized mice after administration of testing

enzymes, and injured tails were immediately placed into PBS (Figure 5A and 5B). The bleeding time until cessation of bleeding was significantly longer in the treatments with plasmin, SK, UK, or tPA compared with the saline control but not in HtrAs (Figure 5C and 5D). Similar results were observed with significant bleeding volume in the treatments with plasmin, SK, UK, or tPA but not in HtrAs (Figure 5E and 5F). Although hemoglobin contents in plasmin, SK, UK, or tPA-treated groups were higher than the control, the hemoglobin contents in HtrAs were similar to the control (Figure 5G and 5H). At the end of the bleeding experiments, the anticoagulation effect was investigated by measuring the clotting times of the mice (Figure 5I and 5J). Blood clotting time was determined by the time for clotting of a drop of fresh blood so that it cannot be elevated from a glass slide. In accordance with the bleeding data, the clotting times were significantly increased in the treatments with plasmin, SK, UK, or tPA compared with the control but not in HtrAs treatments. There was no significant difference in tail bleeding and clotting time between the control and the HtrAs treatment groups. A rebleeding assay was performed to observe the clot stability after the intravenous administration of thrombolytics. There was no difference in rebleeding times between the control and HtrA groups, indicating significantly higher clot stability in HtrA treatment compared to the cotested thrombolytics (Figure 5K and 5L).

HtrAs Precisely Dissolved Intravascular Thrombi In Vivo With the Complete Rescue of Thrombosis and Embolism in Mice

The in vivo thrombolytic efficacies of HtrAs were examined using the κ -carrageenan-induced thrombosis mouse model by injecting 20 mg/kg of κ -carrageenan to each mouse (Figure VI in the [Data Supplement](#)). Although all tested thrombolytics were able to reduce thrombus length as well as the degree of redness and swelling in the thrombotic tails, HtrAs were incomparably superior in thrombolytic efficacies than other thrombolytics (Figure 6A through 6D; and Table II in the [Data Supplement](#)). The length and the ratio of thrombus were most reduced by HtrA treatments, significantly different from those of plasmin, SK, UK, or tPA treatment. In addition to the visual observation of the thrombotic tails (Figure 6C and 6D), the histological examinations were also taken (Figure 6E and 6F). As expected, the control group displayed multiple sites

Figure 3 Continued. An excisional full-thickness wound ($\approx 30 \text{ mm}^2$) was made in the tail skin of BALB/c mice. The excised wounds were incubated at 37 °C for 72 h with either 50 mmol/L Tris-HCl as Ctrl or indicated thrombolytics ($n=3$). The wound healing was observed by measuring the turbidity at 550 nm as shown with the representative visual images. **I** and **J**, Characterization of cross-linked fibrin products in thrombolytics-treated wound tissue using an anti-fibrin α -chain specific monoclonal antibody. Arrow indicates the digested products. Error bars, mean \pm SD. Data (**A**, **B**, **G**, and **H**) were analyzed using 2-way ANOVA with Tukey multiple comparison test based on the normality of data assessed by the Shapiro-Wilk normality test. *P* values (**G** and **H**) of (i), (ii), (iii), and (iv) represent the significance level between HA1/HA2 vs Ctrl, UK (urokinase)/tPA (tissue-type plasminogen activator), SK (streptokinase), or PL (plasmin) at 30 h, respectively. NS indicates not significant.

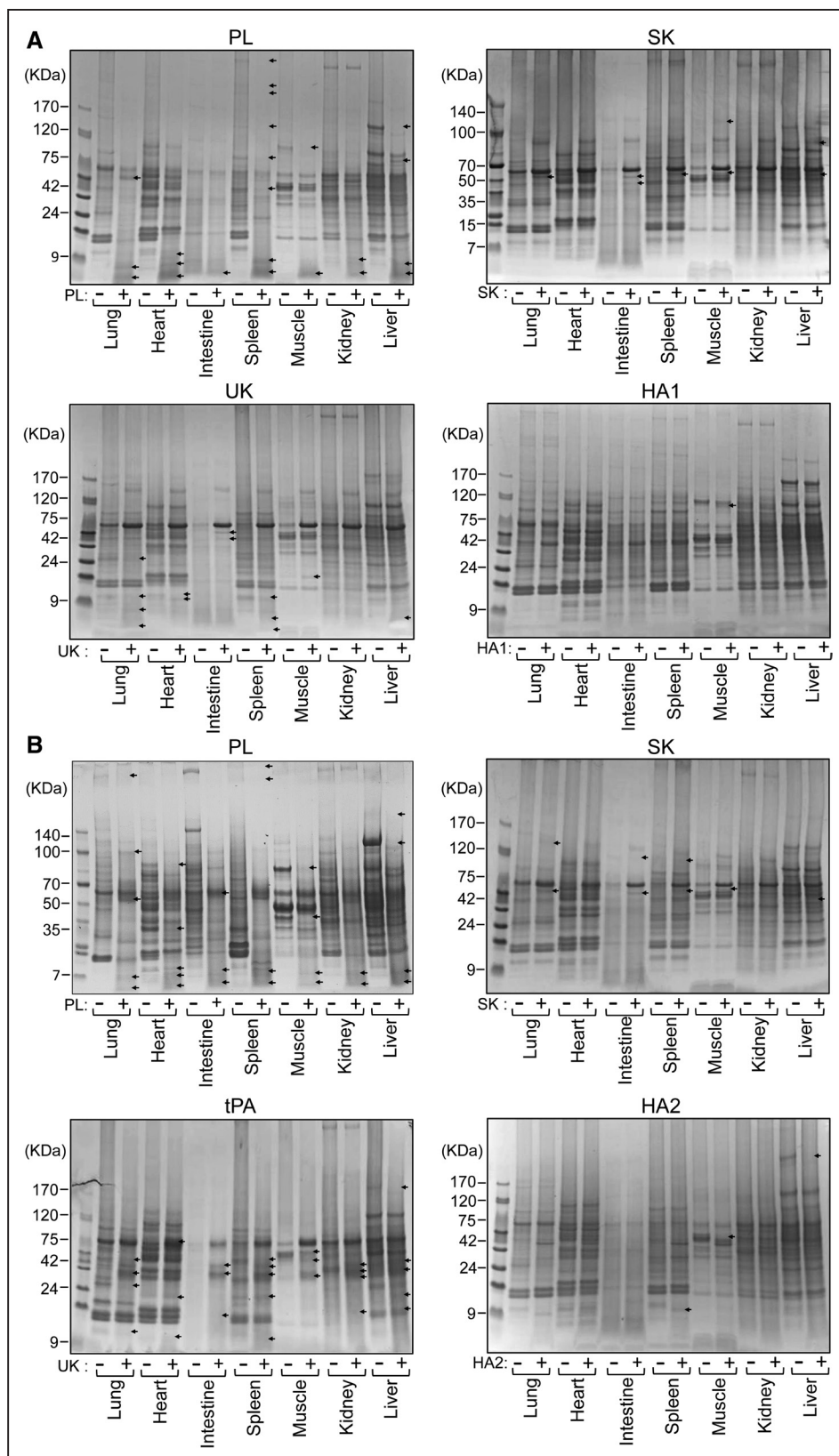


Figure 4. HtrAs (high-temperature requirement As) resulted in the least degree of nonspecific proteolysis compared with other thrombolytic enzymes.

Total proteins (10 µg) from various mouse tissues (**A** and **B**), human tissues (**C** and **D**), and platelet cells (**E**) were incubated in the presence (+) or absence (–) of thrombolytics as indicated. Samples were separated on 4%–12% SDS-PAGE gel followed by Coomassie blue staining. Arrows indicate changes in the protein bands by nonspecific protein degradation with each enzyme treatment. Ctrl indicates control; HA1, HtrA1; HA2, HtrA2/Omi; MRK, marker; PL, plasmin; SK, streptokinase; tPA, tissue-type plasminogen activator; and UK, urokinase. (Continued)

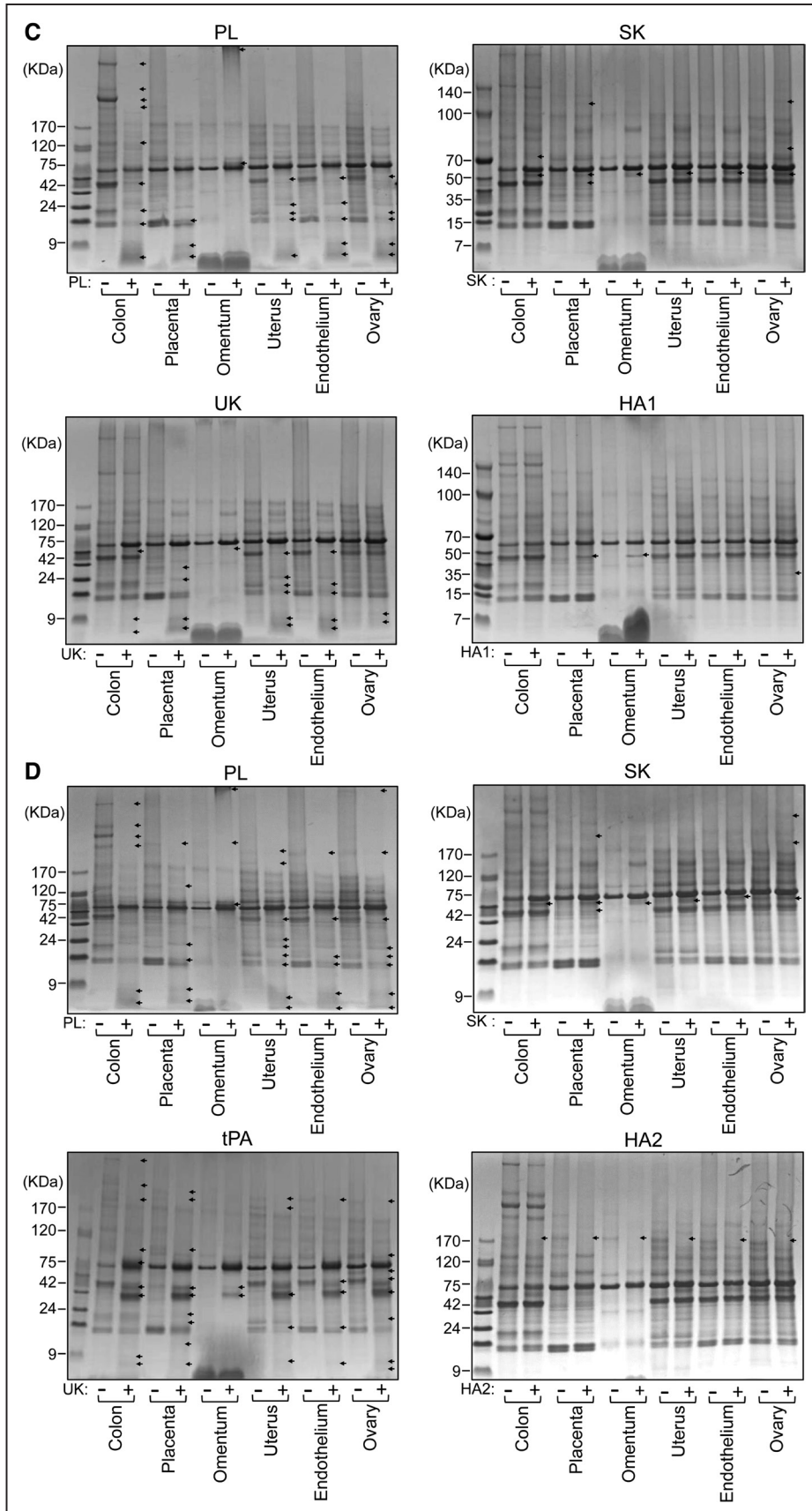


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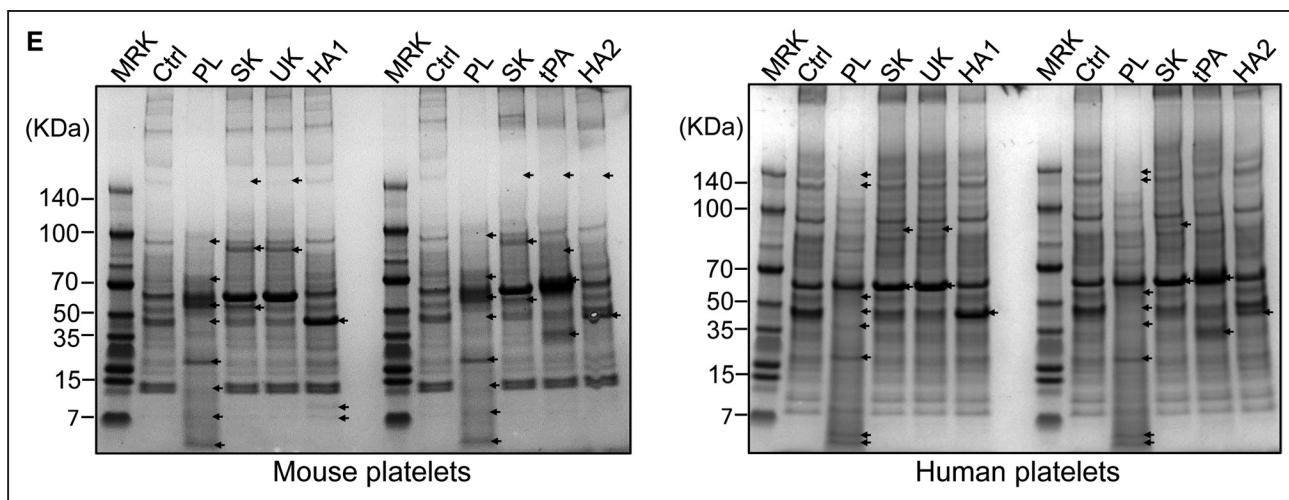


Figure 4 Continued.

of blood clamping throughout the tissue, whereas tested thrombolytics moderately reduced the degrees of clamping. In HtrA treatments, however, intravascular blood clamping was completely absent in agreement with the visual observation of thrombotic tails. Clearly, HtrAs effectively dissolved thrombi in mouse tails (Figure 6A through 6F) in a dose-dependent manner (Figure VII in the [Data Supplement](#)). Furthermore, HtrA treatment reduced the expression of inflammatory marker CD68 in thrombosis tail (Figure 6G and 6H).

The effect of HtrAs on thrombosis was further evaluated in an ADP-induced pulmonary embolism mouse model (Figure 6I and 6J). Although no mice survived in saline control, the survival rates with treatments of plasmin, SK, UK, and HtrA1 were 50%, 33.33%, 66.67%, and 100%, respectively (Figure 6I). Similarly, the survival rates with treatments of saline, plasmin, SK, tPA, and HtrA2 were 0%, 60%, 20%, 40%, and 100%, respectively (Figure 6J). Among tested thrombolytics, only HtrA treatments resulted in complete protection with 100% survival against ADP-induced pulmonary embolism (Figure 6G and 6H; and Table III in the [Data Supplement](#)). The complete rescues of pulmonary thromboembolism in mice demonstrate that HtrAs are ideal thrombolytic enzymes that can effectively remove intravascular thrombi to prevent and/or treat thrombosis without any serious complications.

To investigate the thrombolytic efficacy of HtrAs against a stable thrombus, carotid arterial thrombi were incubated with thrombolytics (IV 40 mg/kg) after carotid arterial injury induced by FeCl_3 (Figure VIII in the [Data Supplement](#)). Intravenous administration of HtrAs significantly lowered the level of thrombus area unlike cotested thrombolytics (Figure 7A and 7B). Furthermore, HtrAs treatment reduced the inflammatory status of the carotid artery (Figure 7C through 7F).

Next, we evaluated the acute toxicity of HtrAs. LD_{50} of HtrAs could not be determined in this study because

the highest doses of HtrA treatments, 1750 mg/kg dose with HtrA1, and 2000 mg/kg dose with HtrA2/Omi did not result in any observable death with BALB/c mice.

DISCUSSION

Thrombosis caused by the formation of intravascular thrombi is one of the biggest medical challenges currently.³² Because of the significantly high incidence rate as well as its fatal consequences, thrombolytic enzymes have been extensively investigated throughout the past several decades.^{4–6} The risks associated with current plasmin-dependent thrombolytics, however, have restricted their application in the treatment of thrombosis.³³ The nonspecific proteolytic activity of plasmin-dependent thrombolytics causes widespread tissue damage and bleeding by degrading a wide variety of normal proteins, especially in blood vessels. In addition, it disturbs the wound healing processes by degrading fibrinogen and FN as well as activating plasminogen to proteolytic plasmin.^{34–36}

In this study, HtrA proteins were shown to preserve both fibrinogen and FN without activating plasminogen to plasmin (Figures 1 through 4). Contrary to the current thrombolytics, HtrAs did not have undesired nonspecific proteolytic activities (Figures 3 and 4). It seems that the specificities of HtrAs enable them to selectively remove intravascular thrombi without affecting untargeted wound thrombi. Animal model experiments confirmed that the outstanding thrombolytic activities of HtrAs precisely dissolved blood thrombi without disturbing the wound healing processes (Figures 5 and 6), suggesting mammalian HtrAs, preferably HtrA1 and HtrA2/Omi, as the first thrombolytic agents with specific activities for intravascular thrombi without undesirable side effects. Our findings demonstrate that HtrAs induce thrombus degradation through specific fibrinolysis and dis-aggregation of platelets without bleeding time prolongation, through a

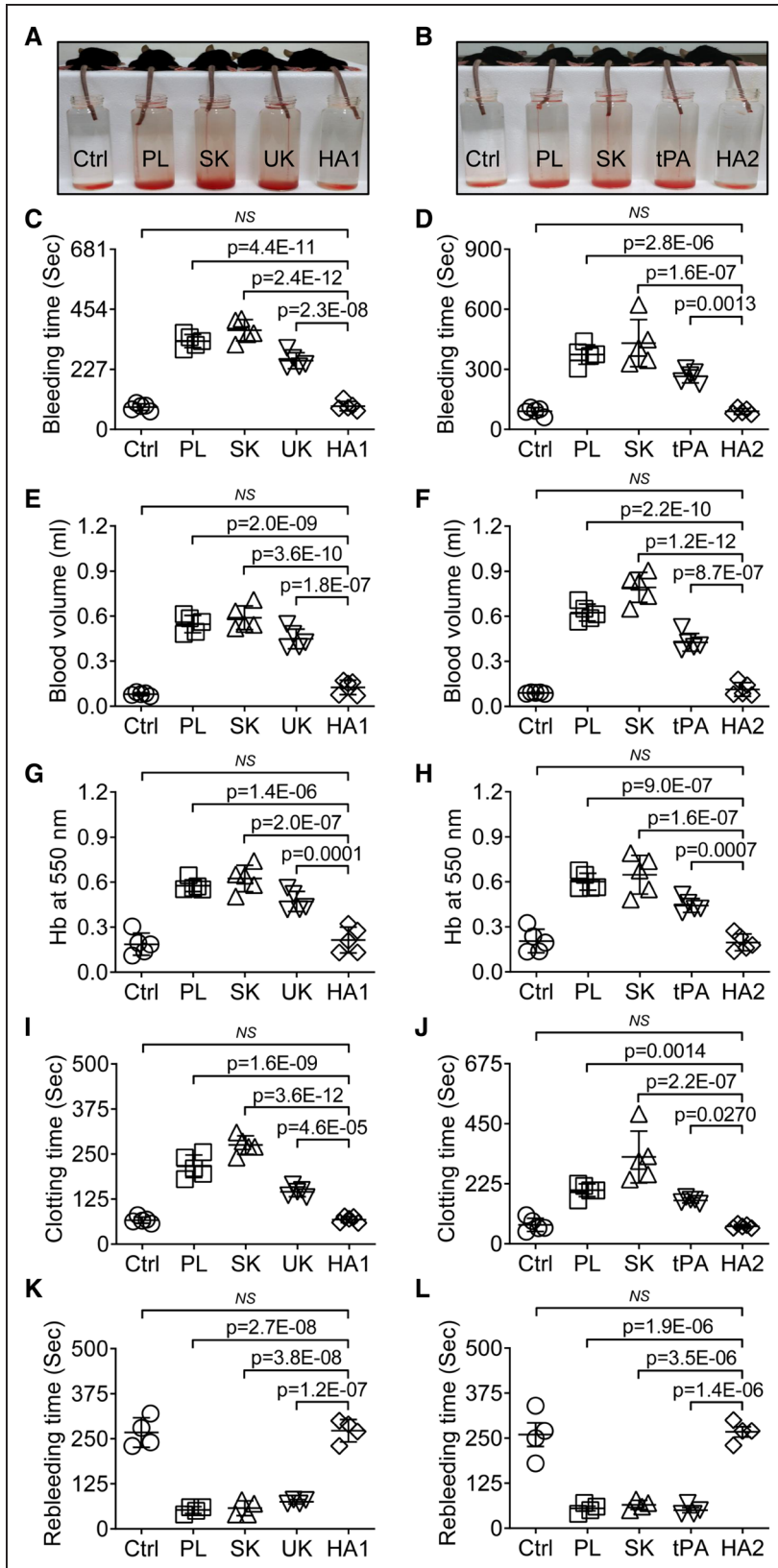


Figure 5. In vivo effect of HtrAs (high-temperature requirement As) on tail bleeding and rebleeding. **A** and **B**, Tail bleeding assay performed with 15-week old C57BL/6 female mice. Either PBS as control (Ctrl) or the thrombolytics as indicated were injected intravenously at 40 mg/kg dose to each group (n=5) of mice. After 30 min treatment, the tails were amputated from anesthetized mice and their blood was collected until the cessation of bleeding. **C** and **D**, The bleeding time (n=5). **E** and **F**, The bleeding volume (n=5). **G** and **H**, The hemoglobin content in the collected blood (n=5). **I** and **J**, The clotting time for coagulation of the blood drops of the punctured tail veins (n=5). **K** and **L**, A rebleeding time measured as the time between the cessation of the bleeding and the start of the second bleeding (n=4). Error bars, mean±SD. Data (**C** through **L**) were analyzed using 1-way ANOVA with Tukey multiple comparison test based on the normality of data assessed by either the Shapiro-Wilk (**C, D, E, F, H, I, J, K, and L**) or Kolmogorov-Smirnov (**G**) normality test. HA1 indicates HtrA1; HA2, HtrA2/Omi; HB, hemoglobin; NS, not significant; PL, plasmin; SK, streptokinase; tPA, tissue-type plasminogen activator; and UK, urokinase.

thrombolytic mechanism independent from the classical fibrinolytic pathway.

HtrAs belong to ATP-independent serine protease family proteins that play important roles in various cellular

processes including cell growth, cell signaling, invasion, inflammation, and apoptosis through substrate modification.^{37,38} Both HtrA1 and HtrA2/Omi are homologs of the bacterial heat-shock protein HtrA.²⁶ The expression of

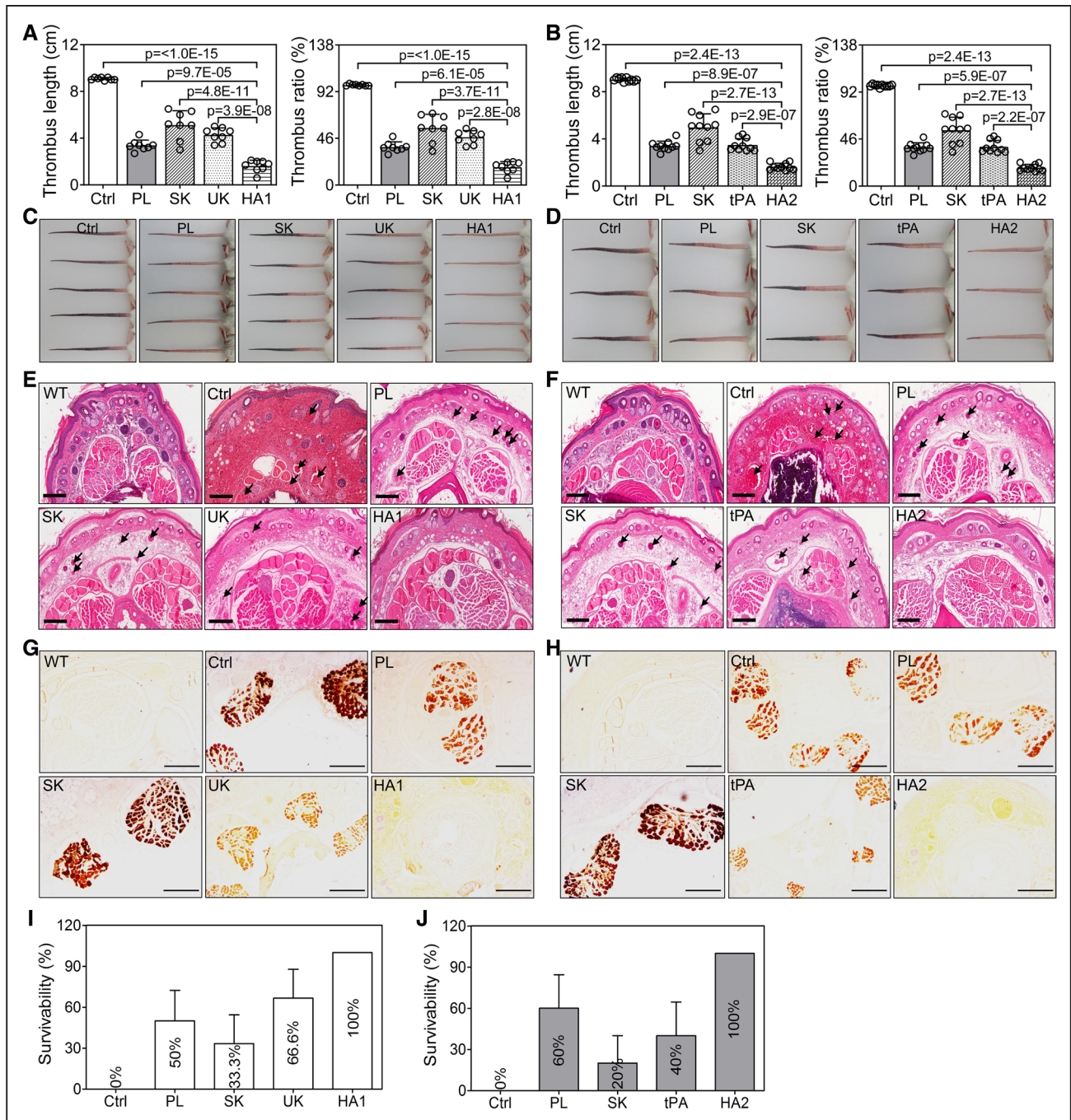


Figure 6. The in vivo thrombolytic efficacies of HtrAs (high-temperature requirement As) on mice tail thrombosis and embolism.

A–H, The κ -carrageenan–induced tail thrombosis model was established with 15-wk-old female BALB/c mice. Either normal saline as control (Ctrl) or indicated thrombolytics were injected intravenously at 40 mg/kg dose to each group (HA1 [HtrA1] group; n=8 or HA2 [HtrA2/Omi] group; n=10) of the mice with tail thrombosis. **A** and **B,** The length of thrombosis measured at 24 h after injection of thrombolytics (**left**). The degree of thrombosis measured by counting the thrombosis ratio at 24 h after the injection of indicated thrombolytics (**right**). **C** and **D,** Representative images of thrombosis tails at 24 h after the injection of indicated thrombolytics. **E** and **F,** Representative H&E stained images of thrombosis tail tissues. The blood clumps are indicated with arrows. Scale bars: 300 μ m. **G** and **H,** Representative immunostaining images of thrombosis in tail tissues. The tissue samples were immunostained for the inflammatory marker CD68 using the mouse anti-CD68 antibody and observed using the DAB substrate. Scale bars: 200 μ m. **I** and **J,** Effect of HA1 and HA2 on the ADP-induced pulmonary embolism. The pulmonary embolism was established in the 15-wk-old female C57BL/6 mice by injecting ADP (150 mg/kg). Either normal saline as the Ctrl or thrombolytics were injected intravenously at 40 mg/kg dose to each group (HA1 group; n=6 and HA2 group; n=5) of the mice 30 min before ADP administration. The bar diagrams of mean \pm SEM with error bars represent the survivability of the mice. Data (**A** and **B**) were analyzed using 1-way ANOVA with Tukey multiple comparison test based on the normality of data assessed by the D'Agostino–Pearson normality test. Error bars, mean \pm SEM or SD. PL indicates plasmin; SK, streptokinase; tPA, tissue-type plasminogen activator; UK, urokinase; and WT, wild type.

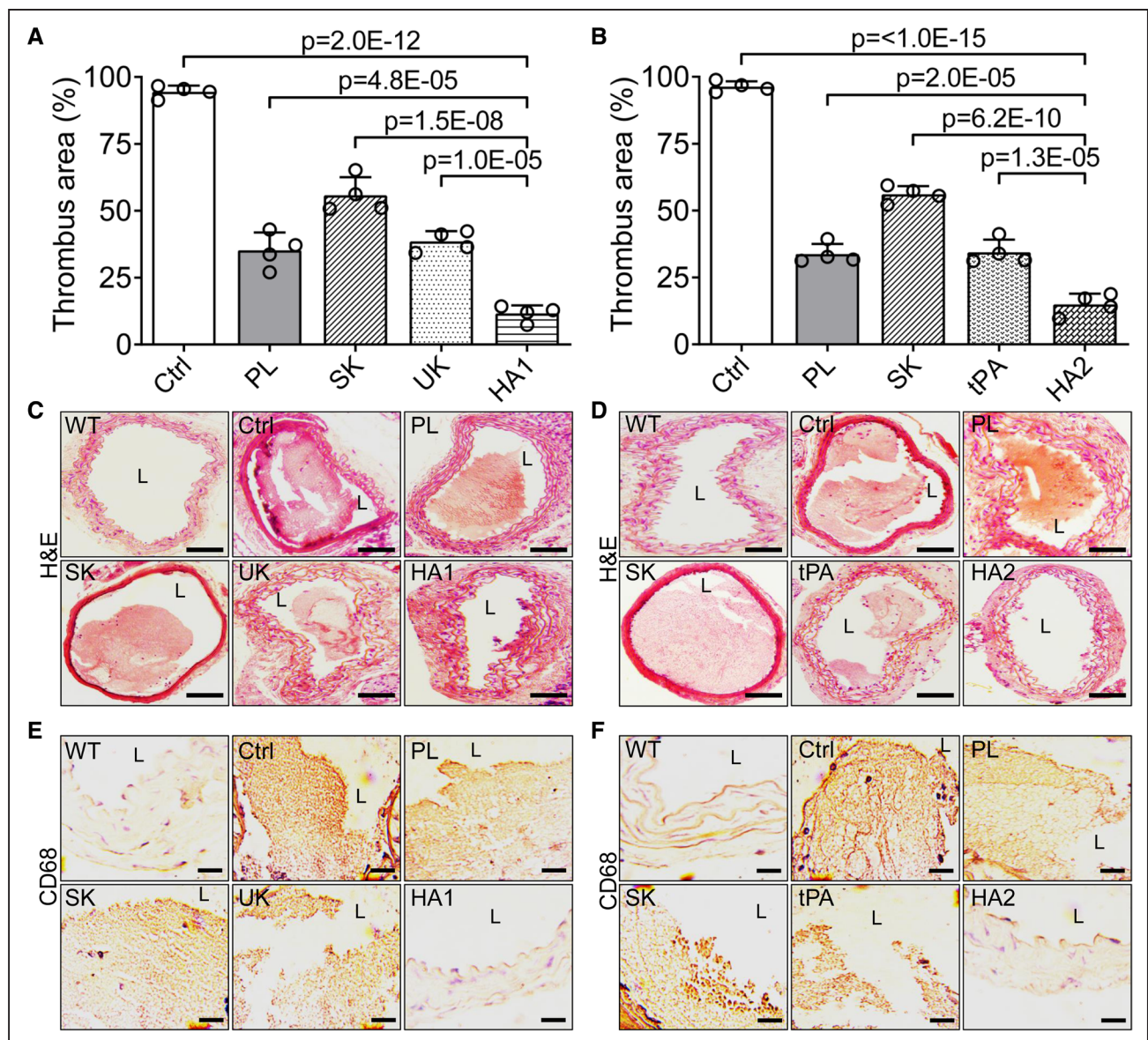


Figure 7. The in vivo thrombolytic efficacies of HtrAs (high-temperature requirement As) on the carotid artery mice model.

A carotid arterial mice model was induced in 12-wk-old female C57BL/6 mice by exposing FeCl_3 on the surface of the right carotid artery after a surgical incision. After FeCl_3 exposure, either normal saline or indicated thrombolytics were injected intravenously at 40 mg/kg dose to each group ($n=4$) of the mice with arterial thrombosis. **A** and **B**, Thrombus ratio in enzyme-treated carotid arteries was quantified from photographs of injured carotid arteries and represented with bar diagram as %. **C** and **D**, Representative hematoxylin and eosin (H&E) stained images of thrombosis in carotid arterial tissues. Scale bars: 200 μm . **E** and **F**, Representative immunostaining images of thrombosis in carotid arterial tissues. The tissue samples were immunostained for the inflammatory marker CD68 using the mouse anti-CD68 antibody and observed using the DAB substrate. Scale bars: 400 μm . Error bars, mean \pm SD. Data (**A** and **B**) were analyzed using 1-way ANOVA with Tukey multiple comparison test based on the normality of data assessed by the Shapiro-Wilk normality test. Ctrl indicates control; HA1, HtrA1; HA2, HtrA2/Omi; L, lumen; PL, plasmin; SK, streptokinase; tPA, tissue-type plasminogen activator; UK, urokinase; and WT, wild type.

HtrA is highly upregulated in bacteria so that HtrA specifically recognizes and degrades denatured proteins when the surrounding temperature is elevated.^{21,39,40} Therefore, HtrA is an essential life-supporting enzyme for bacteria. Interestingly, HtrA is evolutionarily well-conserved regarding its amino acid sequence and the 3-dimensional structure.^{25,27} Considering that the bacterial function of HtrA is to degrade misfolded proteins through its proteolytic activity, it is very interesting to note that the function of HtrA1

and HtrA2/Omi are evolutionarily conserved in animals to remove denatured proteins, such as intravascular thrombi.

The expression of human serine protease HtrA1 is ubiquitous in adult tissues.⁴¹ The peptidase activity of the serine protease domain is indispensable for its role in protein quality control, as well as cell signaling, cell growth, apoptosis, etc.^{42,43} For protein quality control, HtrAs also contain a PDZ (post synaptic density protein [Psd-95]) domain, which is known for its role

in recognizing substrates with a structural abnormality, such as misfolding or aggregation. This study suggests that the PDZ domain of HtrAs might selectively recognize the abnormally-formed fibrin clots in intravascular thrombi, followed by proteolytic degradation of thrombi with serine protease domain of HtrAs. Mutations in serine protease domain or other coding regions of HtrA1 have been reported in cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy, an inherited form of cerebral small vessel disease which is the leading genetic cause of recurrent lacunar ischemic stroke and cognitive impairment in young adults.^{44–46} In this context, this work provides an explanation to the underlying association between HtrA1 and cardiovascular diseases. Also, this work demonstrates that HtrA1 and HtrA2/Omi are ideal thrombolytic agents for the treatment of various vascular occlusive diseases where hemorrhagic complications of current thrombolytic agents are a major concern.

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Disclosures

None.

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