Saxatilin, a Novel Disintegrin as a New Drug Candidate for Thrombolytic Therapy

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Saxatilin, a Novel Disintegrin as a New Drug Candidate for Thrombolytic Therapy

Directed by Professor Ji Hoe Heo

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Il Kwon

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This certifies that the Doctoral Dissertation of Il Kwon is approved.

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Finally, I simply hope that my devotion to medical science could one day open doors to those who seek genuine help.

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ABSTRACT

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Saxatilin, a novel disintegrin purified and cloned from Korean snake (*Gloydius saxatilis*) venom, strongly inhibits aggregation and activation of platelets by inhibiting multiple integrins such as $\alpha_{2b}\beta_3$ (GP IIb/IIIa), $\alpha_5\beta_1$, and $\alpha_v\beta_3$. The thrombolytic effect of saxatilin in mice was investigated using the ferric chloride-induced carotid arterial thrombosis model. Thrombotic occlusion and resolution of the thrombus were assessed by measuring blood flow in the carotid artery with an ultrasonic flow meter and confirmed by histological observations. Thrombolytic patterns of rt-PA, u-PA, and abciximab were investigated and compared to the thrombolytic pattern of saxatilin. The

thrombolytic effectiveness of saxatilin on the aged thrombus were also assessed and compared with the effectiveness of rt-PA. Saxatilin dissolved the thrombus in a dose-dependent manner. Blood flow was notably restored at a dose of 2.5 mg/kg, significantly restored at a dose of 3.75 mg/kg, and nearly reached the baseline level at a dose of 5 mg/kg. As the dose of saxatilin increased, the time to recanalization decreased. Among several regimens tested, a bolus injection of 10% of the dose and continuous infusion of the remaining dose for one hour resulted in effective recanalization without reocclusion. Thrombolytic therapy by saxatilin showed a stronger efficacy, a shorter time to effective recanalization, and a lower frequency of reocclusion than by rt-PA, u-PA, and abciximab. The effectiveness of thrombolytic therapy by both saxatilin and rt-PA decreased as thrombus age increased. Bleeding complications were observed in two of 51 mice that received saxatilin. Fibrin/fibrinogen zymography and platelet aggregometry studies indicated that saxatilin exerts its action on platelets. Saxatilin, which inhibits multiple integrins acting on platelets, was safe and effective in resolving ferric chloride-induced thrombus in mice.

Key words: Saxatilin, Ferric chloride-induced arterial thrombosis, Thrombolysis, Anti-aggregation receptor therapies

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I. INTRODUCTION

Intravenous administration of recombinant tissue plasminogen activator (rt-PA) is effective in treating ischemic strokes within 4.5 hours of symptom onset.[1, 2] However, more than two-thirds of patients fail to achieve successful recanalization after IV rt-PA treatment, and rt-PA appears to be a neurotoxin.[3-5] To improve thrombolytic potency and to reduce potential adverse effects of rt-

PA, several new thrombolytic agents have been developed. They include variants of t-PA, microplasmin, plasmin, and plasminogen activators from animal sources.[6-9] These drugs aim to enhance fibrin specificity, extend plasma half-life, reduce inhibition by plasminogen activator inhibitor-1, and avoid neurotoxicity. While these drugs target fibrin of the thrombus, the thrombus is formed by platelet-fibrinogen interaction. Resistance of the platelet-rich thrombi to thrombolytic agents targeting the fibrin is one of the primary causes of thrombolysis failure. In this regard, treatment targeting platelets may be useful, and disaggregation of platelets from fibrin is another potential approach to dissolve thrombi.[10, 11]

Adhesion and aggregation of platelets are mediated by interactions of ligands with multiple integrins, which include integrins $\alpha_{2b}\beta_3$ (GP IIb/IIIa), $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$. Among them, the platelet glycoprotein (GP) IIb/IIIa receptor, which mediates the final common pathway of platelet aggregation by binding specifically to fibrinogen,[12] has been the main target in the development of drugs that act against the platelets. Several platelet GP IIb/IIIa receptor antagonists have been developed, which include the Fab fragment of a human-mouse chimeric antibody against GP IIb/IIIa (abciximab), nonpeptide analogues of an Arg-Gly-Asp (RGD) peptide (tirofiban and lamifiban), and a cyclic

heptapeptide disintegrin containing KGD motif (eptifibatide).[13-15] These GP IIb/IIIa antagonists have been effective for resolution of the thrombus by dethrombotic mechanisms (disaggregation of platelets from already bound fibrinogen) in selected patients with acute coronary syndrome and stroke.[13, 14, 16-18]

Saxatilin, a novel disintegrin purified and cloned from Korean snake (*Gloydius saxatilis*) venom, has the tripeptide sequence RGD, which is a recognition site of disintegrins to a platelet GP IIb/IIIa receptor.[19, 20] Saxatilin, with a molecular mass of 7712 Da, strongly inhibits platelet aggregation[20] and platelet activation[21] by inhibiting multiple integrins such as $\alpha_{2b}\beta_3$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$. Considering the known effects of GP IIb/IIIa receptor inhibitors on thrombus resolution, it is possible that saxatilin may possess thrombolytic effects. In this study, the effect of IV saxatilin on thrombus resolution in mice was examined using the ferric chloride ($FeCl_3$)-induced arterial thrombosis model.[22, 23]

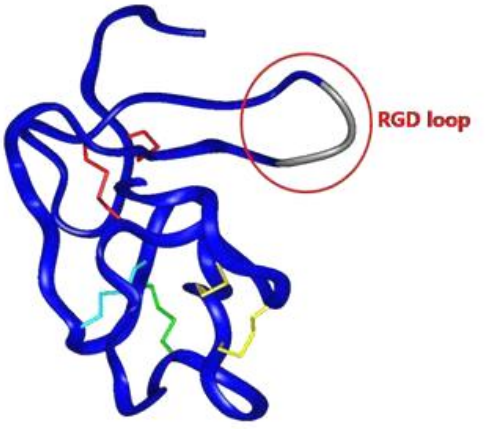

Figure 1. Three-dimensional structure of saxatilin

Figure 2. Disulfide bond pattern of saxatilin[19]

II. MATERIALS AND METHODS

1. Experimental animals and $FeCl_3$-induced carotid artery thrombosis

Eight-week-old male mice from the Institute of Cancer Research (ICR) were used in this study. The care and use of laboratory animals in this experiment were performed according to institutionally approved protocols in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). For operative procedures, the animals were anesthetized by inhalation of 5% isoflurane in a mixture of 70% N_2O and 30% O_2. Anesthesia was maintained with 2% isoflurane. During the operative procedures, body temperature was monitored continuously with a rectal probe and was maintained at 37.0 ± 0.2 ℃ by means of a homeothermic blanket control unit and a heating pad (Harvard Apparatus, Holliston, MA, USA). A $FeCl_3$-induced carotid thrombosis model was used to test the thrombolytic activity of saxatilin in vivo. A midline cervical incision was made, and the left common carotid artery was carefully dissected under a surgical microscope. An ultrasonic Doppler flow probe (MA0.7PSB; Transonic Instruments, Ithaca, NY, USA) was placed around midportion of the common carotid artery (CCA). Carotid blood flow was obtained with a Transonic TS420 Blood Flow Meter (Transonic Instruments, Ithaca, NY, USA) and an iWorx IX-304T data acquisition system

(iWorx Systems, Inc., Dover, NH). Baseline flow of the CCA was measured for five minutes. The ultrasonic Doppler flow probe was removed after the baseline flow was determined. Oxidative vascular injury with chemical stress was induced by placing a filter paper (700 μm × 500 μm) saturated with 50% $FeCl_3$ on the adventitial surface of the midpoint of the exposed CCA for five minutes. After removing the filter paper, the CCA was washed with normal saline and its blood flow was recorded. Thrombus formation and arterial occlusion were determined by decrease of blood flow, and complete occlusion was defined as absence of blood flow for ten minutes.

2. Determination of thrombotic occlusion and measurement of thrombus size

Consistency of the model in formation and size of the thrombus were assessed. Ten minutes after complete occlusion, the injured CCA segments were excised, immediately immersed in 4% paraformaldehyde (PFA) for fixation, and embedded in paraffin for histological analysis. The paraffin blocks were consecutively sectioned in a longitudinal direction into 3-μm-thick slices. The sectioned slices were mounted on glass slides, stained with hematoxylin and eosin, and photographed with a light microscope (Axio Imager.D2; Carl Zeiss Microimaging, Oberkochen, Germany). The size of the thrombus (longitudinal

length and area) in each animal was determined using Zeiss AxioVision software (AxioVs40 V 4.8.1.0; Carl Zeiss imaging Solution GmbH, Germany) in a slice that showed the largest thrombus size.

3. Electron microscopic examination

For transmission electron microscopy (TEM), the arteries were immediately fixed with Karnovsky solution (pH 7.4, 2% glutaraldehyde, 2% paraformaldehyde, 0.5% $CaCl_2$) overnight at 4 ℃, washed in 0.1 M phosphate buffer (pH 7.4) and then post-fixed in 1% osmium tetroxide in the same buffer for two hours. The specimens were then dehydrated through a series of ascending concentrations of ethanol, exchanged through propylene oxide, and incubated with a 1:1 mixture of EPON (EPON 812, MNA, DDSA, DMP 30) and propylene oxide for 18 hours, embedded in a EM oven, and then trimmed. Subsequently, 0.25 μm semi-thin sections were stained with Toluidin blue and observed under a light microscope to determine the $FeCl_3$-damaged region. And after retrimming, ultrathin sections (80 nm) were obtained by ultramicrotome (Ultracut UCT, Leica, Austria) with a diamond knife, double stained with uranyl acetate and lead citrate, and examined in a TEM (JEOL-1011; JEOL, Japan) at 80 kV.[24]

For scanning electron microscopy (SEM), the specimens were fixed and dehydrated in the same manner as described above, replaced with isoamyl acetate, dried in a critical point dryer (HCP-2; Hitachi Co., Tokyo, Japan), coated with a thin layer of gold (100 nm) in an ion coater (IB-3; Eiko engineering, Ibaraki, Japan), and examined with an FE (field emission)-SEM (S-800; Hitachi Co., Tokyo, Japan) at 20 kV.

4. Intravenous thrombolysis using saxatilin

Expression and mass production of saxatilin have been described in previous studies.[19, 25] The total volume of saxatilin for IV administration was 10 μl × body weights (g), and the final doses of saxatilin for IV administration were adjusted with normal saline. Ten minutes after occlusion of the CCA, saxatilin was administered intravenously via the left femoral vein with an infusion pump (KDS100 syringe pump; KD Scientific, Holliston, MA, USA) connected to PE-10 tubing. The carotid blood flow was continuously monitored for two hours from the initial time of injection.

A. Dose response to saxatilin

To evaluate the dose response to saxatilin, the animals were randomly divided into seven groups, with five mice in each group: normal saline (control

group), 1, 1.75, 2.5, 3.75, 5.0, and 10.0 mg/kg of saxatilin administration groups. Ten percent of the dose was administered in bolus intravenously, and the rest was infused continuously for 60 minutes.

B. Methods for administering saxatilin

The thrombolytic effects of saxatilin were assessed for each administration method. For this experiment, a total dose of 5 mg/kg of saxatilin was used in each animal, and the animals were divided into the following four groups, with five mice in each group: 1) bolus injection of total dose (5 mg/kg) at ten minutes after occlusion, 2) double bolus injection of saxatilin with a half dose (2.5 mg/kg) of saxatilin at ten minutes after occlusion and 60 minutes after the first bolus injection, 3) a half dose bolus injection (2.5 mg/kg) at ten minutes after occlusion, and then continuous infusion for 60 minutes of the remaining dose, and 4) bolus injection of 10% of the total dose (0.5 mg/kg) at ten minutes after occlusion, and then continuous infusion of the remaining dose (4.5 mg/kg) for 60 minutes.

5. Assessment of recanalization

The presence and degree of recanalization were assessed by measuring blood flow. Baseline and continuous blood flow data after CCA occlusion were

acquired using iWorx Labscribe 2 data acquisition software (version 2.045000). Immediately after two hours of blood flow monitoring, the CCA was obtained in all mice, fixed with 4% paraformaldehyde solution, and embedded in paraffin for histological examination. The paraffin blocks were consecutively sectioned in a transverse direction into 3-μm-thick slices, mounted on a glass slide, and stained with hematoxylin and eosin.

A. Dose response to saxatilin

Carotid blood flow was determined by calculating the area under the time-flow curves. All measured values were standardized by the minimum blood flow of each animal to avoid differences caused by variations in physiological condition between animals. The thrombolytic effect was calculated as described below and expressed as a percent of mean control baseline blood flow: (mean blood flow during the two hours after administration of saxatilin bolus/ mean baseline blood flow) × 100 (%). The mean values of each group in the dose response study were calculated and graphed on a standard thrombolytic activity curve (mean ± SD).

B. Thrombolytic effects over time

Average blood flow was calculated in each animal every minute to achieve a

representative time-dependent pattern per dose and administration method of saxatilin. The mean values of all animals in each group were calculated, and the temporal changes are shown as continuous bar graphs (Figures 5A/7A).

C. Time to recanalization

Time from administration of saxatilin to effective recanalization was assessed. Effective recanalization was defined as the restoration of blood flow to at least 50% of the baseline level, maintained for longer than 30 minutes.

6. Dose response of rt-PA, u-PA, and abciximab

To evaluate dose responses of rt-PA (Actilyse®; Boehringer Ingelheim, Seoul, Korea), u-PA (Urokinase; Green Cross Corp., Yongin, Korea), and Abciximab (ReoPro®; Lilly Korea, Seoul**,** Korea), the animals were randomly divided into seven groups respectively (five mice in each group): 0 (normal saline), 0.9, 1.8, 2.7, 4.8, 7.2, 9, and 18 mg/kg of rt-PA administration groups, 0 (normal saline), 100, 500, 1,000, 5,000, 10,000, and 50,000 IU/kg of u-PA administration groups, and 0 (normal saline), 0.25, 0.5, 1, 2.5, 5, 10, 20, and 40 mg/kg of abciximab administration groups. Ten percent of the dose was administered in bolus intravenously and the rest was infused continuously for 60 min.

7. Thrombolytic effects on the aged thrombus

Randomly selected five mice were used in each group. Thrombosis was induced in the same manner with that performed in other experiments. To avoid spontaneous recanalization caused by endogenous thrombolytic enzymes or blood pressure, distal end of the CCA was ligated with Silkam 5-0 thread (Silkam, B Braun Aesculap, Tuttlingen, Germany) immediately after complete occlusion. After maturation of the thrombi for 1, 3, 6, 12, and 24 hours, the suture was gently removed. And then 5 mg/kg of saxatilin was administered by bolus injection of 10% of the total dose and then continuous infusion of the remaining dose. The thrombus at a time of complete occlusion was used as control fresh thrombus. Also, 9 mg/kg of rt-PA was administered in the same way after maturation for 3 or 6 hours, to compare with the efficacy of saxatilin.

8. Platelet aggregometry

The inhibitory effect of saxatilin on platelet aggregation was investigated using platelet aggregometry. Blood (900 μl) was drawn by cardiac puncture from five mice anesthetized with isoflurane into a syringe containing 100 μl of 150 USP sodium heparin solution, resulting in a final heparin concentration of 15 USP/ml. A total of 500 μl of heparinized whole blood was mixed with the same volume of normal saline. Different concentrations of saxatilin were

adjusted by dilution with normal saline, and then 0.1, 1, or 2 μg of saxatilin against adenosine diphosphate (ADP) and 5, 50, or 100 μg of saxatilin against collagen were added into each test cuvette and preincubated with magnetic stirring at 37 ℃ for five minutes. The same volume of normal saline was added as a control. Twenty micromolar of ADP or 5 μg/ml of collagen (Chronolog Corporation, Havertown, PA) were used as an agonist for platelet aggregometry. Platelet aggregation activity was measured with an impedance method in a platelet aggregometer (Chronolog 700; Chronolog Corporation, Havertown, PA).

Also, to test the effect of saxatilin on the preformed platelet aggregates, 10, 50, and 250 μg of saxatilin and the same volume of normal saline as a control were added into respective cuvettes after reaching a maximal aggregation in response to 20 μM of ADP or 5 μg/ml of collagen. The effect of platelet disaggregation was calculated as a percentage of restoration to the baseline level.

9. Fibrin/fibrinogen zymography

Fibrin/fibrinogen zymography was performed to determine whether saxatilin has fibrinolytic activity. The fibrinogen gel was prepared using 12% SDS-polyacrylamide gel containing 1.2% fibrinogen (Hyphen Biomed, Neuville-sur-Oise, France). The fibrin gel was prepared by adding 0.1 NIH

unit/ml of thrombin (Hyphen Biomed, Neuville-sur-Oise, France) into fibrinogen gel. A total of 100 μg of saxatilin and 3 ng of rt-PA (Actilyse; Boehringer, Ingel-heim am Rhein, Germany) with an equal volume of sample buffer [80 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 10% glycerol, 0.01% bromophenol blue] were loaded into respective wells of the fibrin/fibrinogen gels. Sample gels were rinsed in 150 ml of 2.5% Triton X-100 (15 min) and incubated with 250 ml of reaction buffer (30 mM Tris, pH 7.4, 200 mM $NaCl_2$, and 0.02% NaN_3) for 12 hours at 37 ℃. After incubation, the gels were stained with 0.1% amido black containing acetic acid, methanol, and distilled water (volume ratio 1:3:6) for one hour and then destained by four washes with the same solution without amido black for 130 minutes. These gels were scanned using a flatbed scanner (ArtixScan F1; Microtek International Inc., Hsinchu, Taiwan).[24, 26]

10. Statistical analysis

All of the statistical computations were performed using SPSS (version 18.0, SPSS Inc, Chicago, IL. USA). The normality of the distribution was verified using the Kolmogorov-Smirnov test. Differences among the groups were compared with a one-way ANOVA test, followed by a post hoc Tukey method. The values were presented as a mean ± standard deviation (SD). $P < 0.05$ was

considered significant.

III. RESULTS

1. The consistency of the animal model

After five minutes of $FeCl_3$ application, the blood flow of the CCA was rapidly and consistently reduced nearly to zero in all five animals examined (Figure 3A). The thrombotic occlusion of the CCA was demonstrated in histologic examinations (Figure 3B). Thrombus size was similar between animals (length: 1.139 ± 0.091 mm, area: 0.305 ± 0.055 mm^2). In the saxatilin-treated groups, histologic examinations did not reveal a thrombus in the carotid lumen of mice with completely restored carotid blood flow, whereas the thrombus remained in the carotid lumen of mice with partially restored carotid blood flow, as well as in mice without any restored carotid blood flow (Figure 4).

2. Electron microscopic features of the carotid artery and thrombus after $FeCl_3$ treatment

The morphological changes in the vascular/blood components as well the composition of thrombus created by $FeCl_3$-induced thrombosis were examined with TEM and SEM. In the region farthest from $FeCl_3$ application, relatively normal vascular structure was observed, and the endothelial cells, smooth

muscle cells, erythrocytes, and discoid platelets were of normal shape (Figures 3Ca/Da). The endothelial cells appeared empty with a loss of organelles. Some platelets were activated, aggregated, and adhered to the luminal surface at the mildly damaged region (Figures 3Cb/Db). At the thrombus border, activated and aggregated platelets adhered to the damaged luminal surface, and no intact endothelial cells were observed (Figures 3Cc/Dc). At the severely damaged region, a platelet-rich thrombus containing erythrocytes completely occluded the vascular lumen (Figures 3Cd/Dd). $FeCl_3$ treatment caused a loss of vascular undulation with a flattened and disconnected arrangement of smooth muscle cells. The internal elastic lamina remained intact even in the severely damaged region.

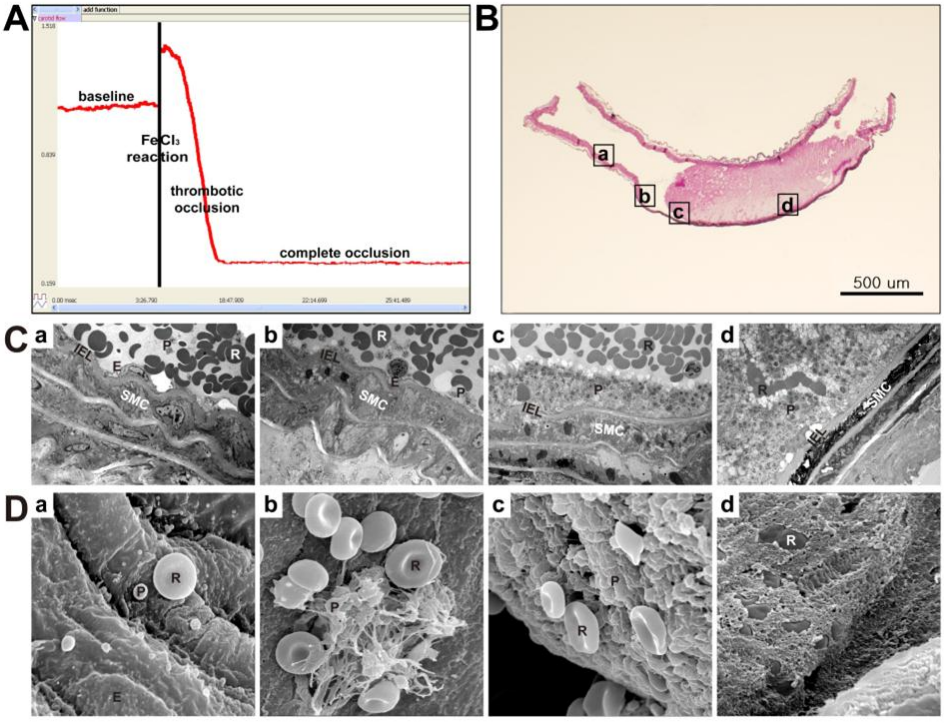

Figure 3. Reliability of the $FeCl_3$-induced arterial thrombosis model. **A:** Blood flow was monitored using an ultrasonic Doppler flow meter in the carotid artery. Representative pattern of the time-flow curve resulting from $FeCl_3$-induced arterial thrombosis. Complete occlusion was defined as absence of blood flow for ten minutes. **B:** Hematoxylin and eosin staining of the carotid artery with thrombotic occlusion after $FeCl_3$ treatment. Original magnification ×100. **C:** Transmission electron microscopy, **(D)** Scanning electron microscopy. The small boxes in Figure 1B indicate sites where electron microscopic photographs were obtained. (a) The region farthest from the site of $FeCl_3$ application; intact vascular structure as well as normally-shaped erythrocytes and discoid platelets are observed. (b) Mildly damaged region. Empty endothelial cells with a loss of organelles, some activated and aggregated platelets, and platelets adhering to the luminal surface were observed. (c) Border region of the thrombus. No intact

endothelial cells were observed. Activated and aggregated platelets firmly adhered to the damaged region of the luminal surface. (d) Severely damaged region. The artery is completely occluded by a platelet-rich thrombus containing several erythrocytes. Also, $FeCl_3$-induced damage caused a loss of vascular undulation, but the internal elastic lamina remains intact even in the severely damaged region. R: erythrocyte, P: platelet, E: endothelial cell, SMC: smooth muscle cell, IEL: internal elastic lamina. Original magnification ×2,000 (TEM), ×5,000 (SEM).

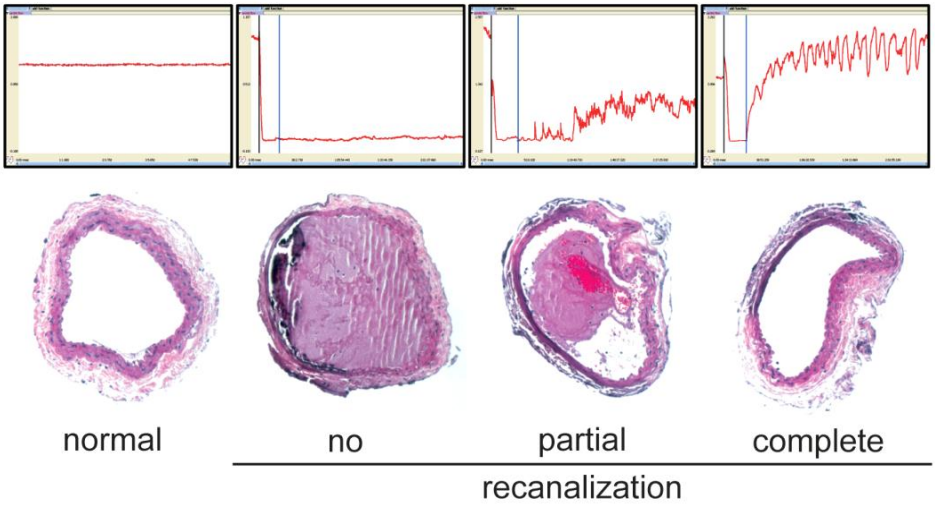

Figure 4. Representative time-flow curves and corresponding histologic features according to the degree of thrombus resolution after saxatilin treatment in the $FeCl_3$-induced carotid artery thrombi of mice. The blood flow is well-correlated with the degree of thrombus resolution. Original magnification ×200.

3. Dose-dependent thrombolytic effects of saxatilin

Dose-dependent effects of saxatilin were determined by calculating the area under the time-flow curve (Figure 5). There were statistically significant differences in thrombolytic effects among the experimental groups ($p<0.001$). Saxatilin treatment did not cause any notable changes at a dose of 1 mg/kg (2.36 ± 0.78%) or 1.75 mg/kg (5.14 ± 2.92%) compared to the status of the normal saline group (2.42 ± 1.07%) (Figure 6). Restored blood flow was observed at a dose of 2.5 mg/kg (32.50 ± 33.70%) and increased in a dose-dependent manner. The restoration of blood flow was significant at a dose of 3.75 mg/kg (60.50 ± 38.78%, $p=0.019$), and the blood flow was restored to nearly baseline levels (94.50 ± 20.47%) at a dose of 5 mg/kg. There was no significant difference between 5 and 10 mg/kg of saxatilin (94.94 ± 39.05%, $p>0.999$).

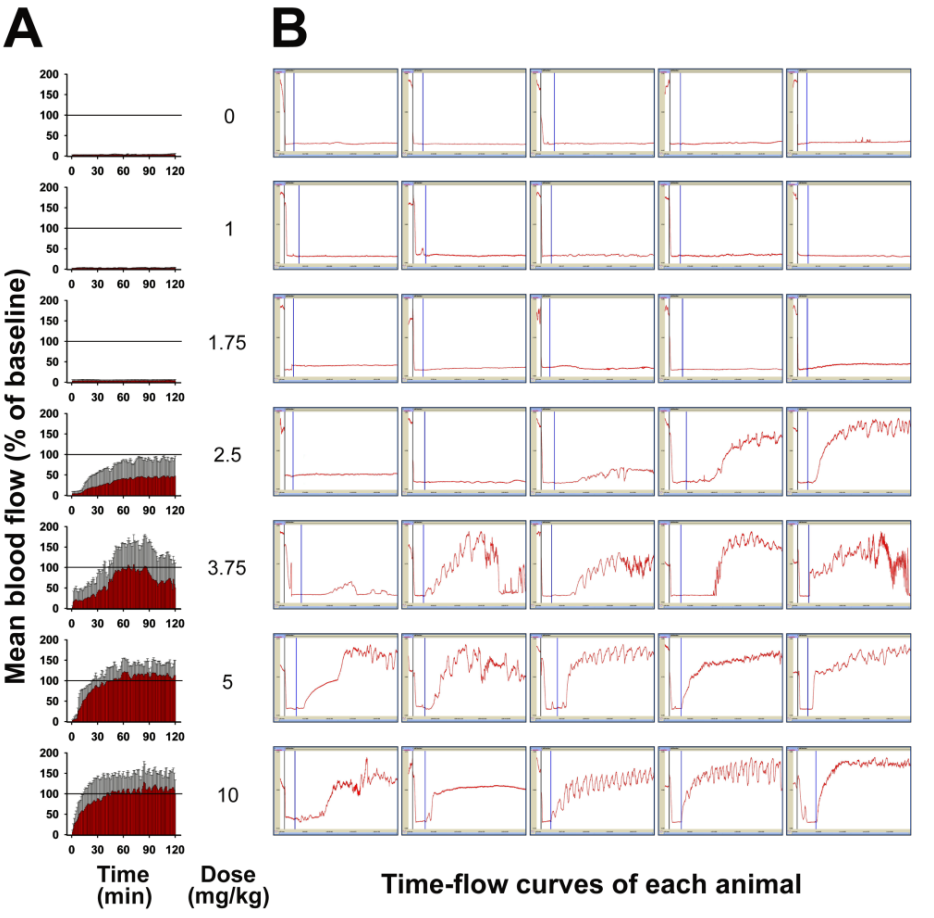

Figure 5. Dose-dependent thrombolytic effect of saxatilin. **A:** The mean values of all animals in each group were calculated, and temporal changes are shown as continuous bar graphs (mean ± SD) **B:** Time-flow curves of all mice in each group. Time to recanalization and frequency of reocclusion are reduced as the dose of saxatilin increases.

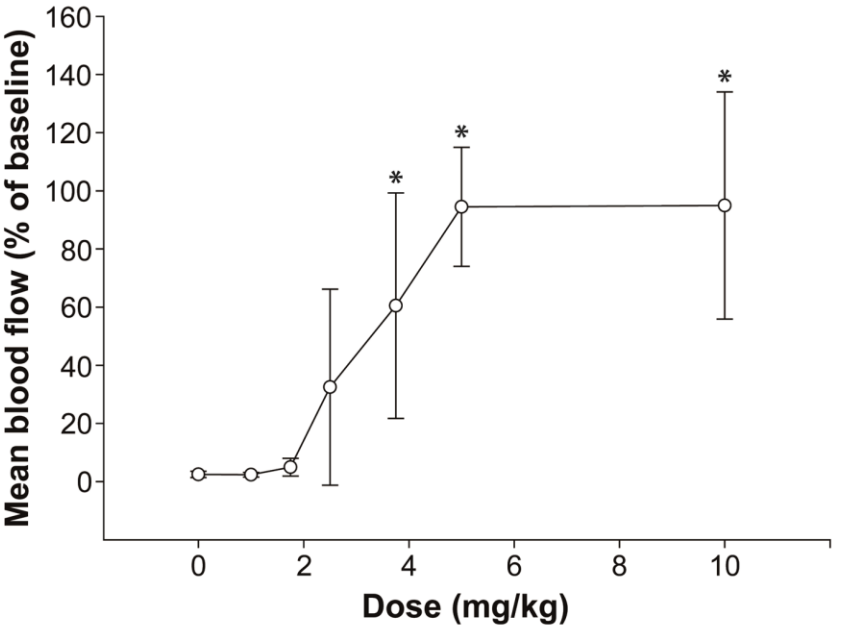

Figure 6. The dose response curve of saxatilin. No notable changes are observed until the dosage reaches 1.75 mg/kg. Restored blood flow is observed at a dose of 2.5 mg/kg, and restoration increases in a dose-dependent manner. Restoration of blood flow is significant at a dose of 3.75 mg/kg ($p=0.019$), and blood flow is restored to nearly baseline levels at a dose of 5 mg/kg. There is no significant difference between 5 and 10 mg/kg of saxatilin ($p>0.999$). *$P<0.05$, significantly different from the control normal saline group.

4. Effectiveness of different saxatilin administration methods

A dose of 5 mg/kg was used to determine the optimal administration method of intravenous saxatilin administration because this dose proved effective in the dose-response study (Figure 5). Mean percentages of blood flow, which were compared to the baseline blood flow, were 77.01 ± 46.11% in the group with a bolus injection of a total dose, 85.23 ± 29.95% in the group with double bolus injection with a half dose, 80.72 ± 30.13% in the group with a half dose bolus injection and continuous infusion of the remaining dose, and 94.50 ± 20.47% in the group with a bolus injection of 10% of the total dose and continuous infusion of the remaining dose.

Decreased thrombolytic effects were observed at different times according to the administration method. Abrupt reocclusion was observed approximately 50 minutes after the first bolus injection in mice treated with double bolus injection, approximately 100 min after a total dose bolus injection, and approximately 110 min after a half-dose bolus injection and continuous infusion of the remaining half dose. Reocclusion was not observed with a bolus injection of 10% of the total dose and continuous infusion of the remaining dose (Figure 7).

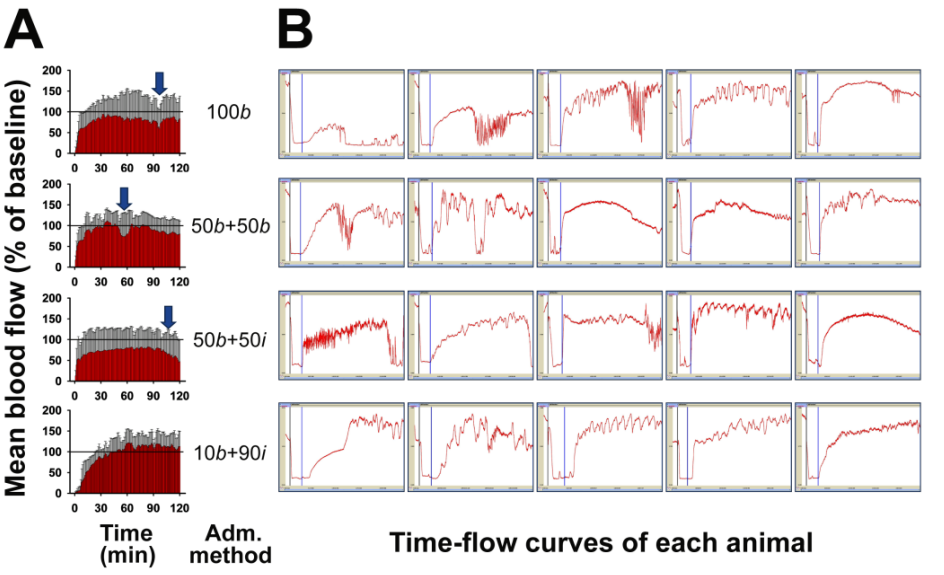

Figure 7. Thrombolytic effect of saxatilin according to administration method. **A:** The time-patterns of blood flow following saxatilin administration. Abrupt reocclusion is observed in mice treated with a bolus injection of 50% of the total dose. Reocclusion is not observed with bolus injection of 10% of the total dose and continuous infusion of the remaining dose. **B:** Time-flow curves of all mice in each group. *b*, bolus injection; *i*, continuous infusion.

5. Time to effective recanalization by saxatilin

Effective recanalization was not observed in mice treated with normal saline, 1 mg/kg of saxatilin, or 1.75 mg/kg of saxatilin. Only two of five mice treated with 2.5 mg/kg of saxatilin and three of five mice treated with 3.75 mg/kg of saxatilin achieved effective recanalization. Effective recanalization was observed in all mice treated with saxatilin at a dose of 5 or 10 mg/kg. Time to effective recanalization was 32.92 ± 23.52 min in mice treated with 2.5 mg/kg saxatilin, 21.75 ± 21.62 min in mice treated with 3.75 mg/kg, 13.92 ± 6.02 min in mice treated with 5 mg/kg, and19.46 ± 19.75 min in mice treated with 10 mg/kg of saxatilin (Figure 8A).

Effective recanalization was also evaluated according to saxatilin administration method. All mice treated with 5 mg/kg of saxatilin achieved effective recanalization except two mice administered a bolus injection of the total dose. Time to effective recanalization was 2.86 ± 0.22 min in mice treated with a bolus injection of the total dose, 13.44 ± 26.31 min with a double bolus injection, 19.48 ± 25.94 min with a half dose bolus injection followed by continuous infusion of the other half dose and 13.92 ± 6.02 min with a bolus injection of 10% of the total dose and continuous infusion of the remaining dose (Figure 8B).

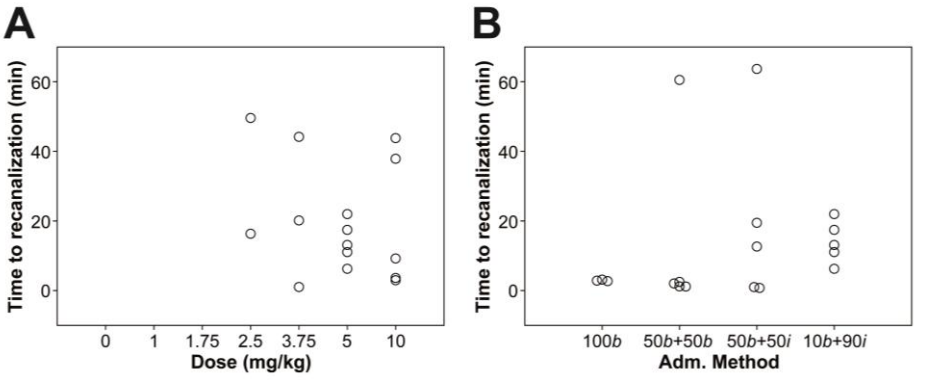

Figure 8. Time to effective recanalization. Effective recanalization was defined as restoration of blood flow to at least 50% of the baseline level, maintained for longer than 30 minutes. **A:** Time to effective recanalization according to administration dose. There is no effective recanalization observed at a dose of 0, 1, or 1.75 mg/kg. Two mice in 2.5 mg/kg and three mice in the 3.75 mg/kg administration groups achieved effective recanalization. All mice treated with 5 or 10 mg/kg achieved effective recanalization. **B:** Time to effective recanalization according to administration method. All mice except two mice administered a bolus injection of the total dose achieve effective recanalization. *b*, bolus injection; *i*, continuous infusion.

6. Dose-dependent thrombolytic effects of rt-PA, u-PA, and abciximab

A. Dose-dependent thrombolytic effects of rt-PA

Dose-dependent effects of rt-PA were determined by calculating the area under the time-flow curve (Figure 9). There were statistically significant differences in thrombolytic effects among the experimental groups ($p<0.001$). rt-PA treatment did not cause any notable changes at a dose of 0.9 mg/kg (8.40 ± 7.16%) and at a dose of 2.7 mg/kg (15.43 ± 8.04%), compared to the status of the normal saline group (2.47 ± 1.07%) (Figure 10). The restoration of blood flow was significant at a dose of 7.2 mg/kg (54.45 ± 21.68%, $p=0.001$), and increased in a dose-dependent manner. The blood flow was restored to nearly baseline levels at a dose of 9 mg/kg (80.52 ± 20.16%). There was no significant difference between 9 and 18 mg/kg (92.44 ± 27.27%) of rt-PA ($p>0.999$).

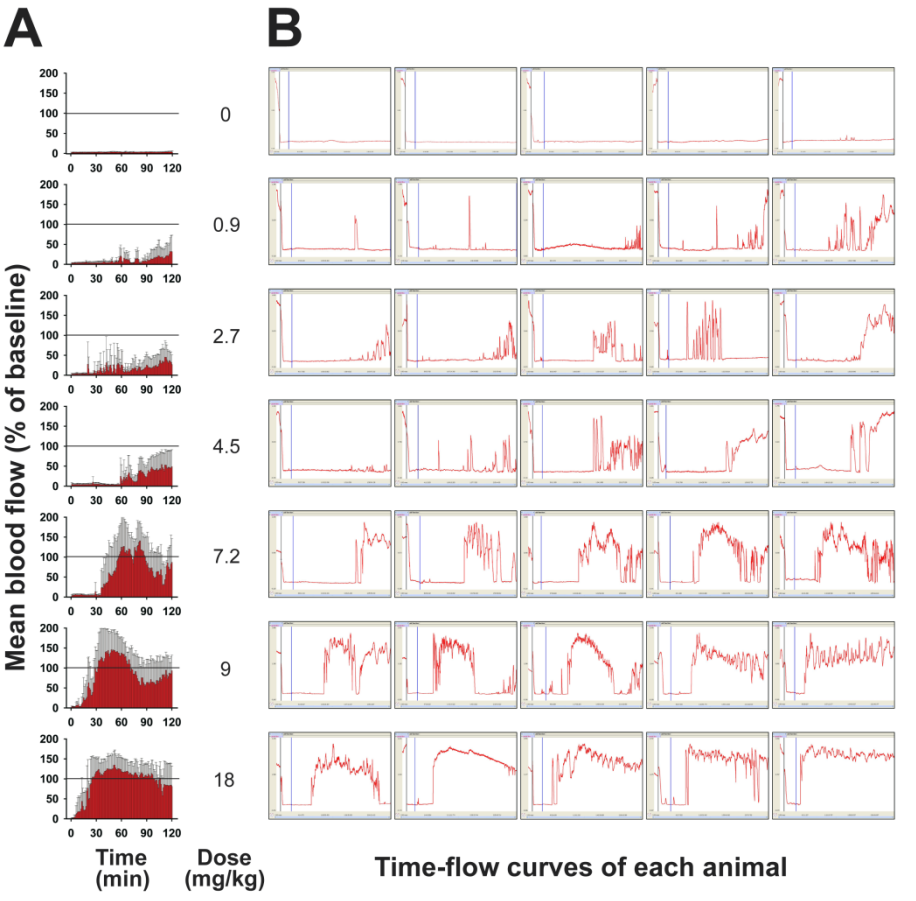

Figure 9. Dose-dependent thrombolytic effect of rt-PA. **A:** The mean values of all animals in each group were calculated, and temporal changes are shown as continuous bar graphs (mean ± SD) **B:** Time-flow curves of all mice in each group. Time to recanalization and frequency of reocclusion are reduced as the dose of rt-PA increases.

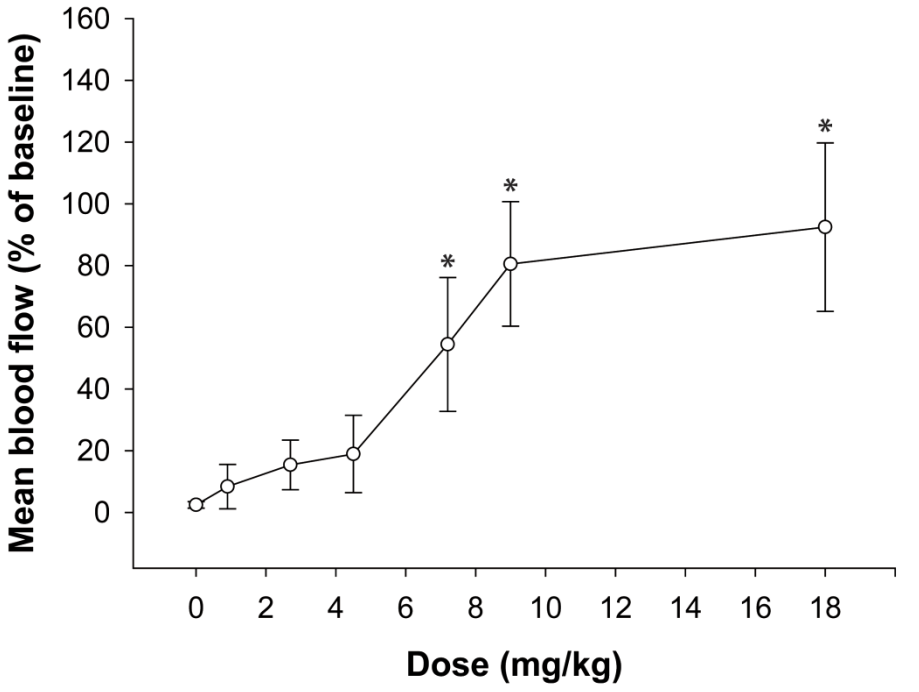

Figure 10. The dose response curve of rt-PA. No notable changes are observed until the dosage reaches 4.5 mg/kg. Restoration of blood flow is significant at a dose of 7.2 mg/kg ($p=0.001$), and blood flow is restored to nearly baseline levels at a dose of 9 mg/kg. There is no significant difference between 9 and 18 mg/kg of rt-PA ($p>0.999$). *$P<0.05$, significantly different from the control normal saline group.

B. Dose-dependent thrombolytic effects of u-PA

Dose-dependent effects of u-PA were determined by calculating the area under the time-flow curve (Figure 11). There were statistically significant differences in thrombolytic effects among the experimental groups (p=0.004). u-PA treatment did not cause any notable change at a dose of 100 IU/kg (4.28 ± 4.33%) and at a dose of 500 IU/kg (7.66 ± 1.91%), compared to the status of the normal saline group (2.47 ± 1.07%) (Figure 12). Restored blood flow was observed at a dose of 1,000 IU/kg (17.41 ± 14.96%), and increased in a dose-dependent manner. The restoration of blood flow was significant at a dose of 10,000 IU/kg (34.96 ± 25.74%, p=0.049). There was no significant difference between 10,000 and 50,000 IU/kg (35.68 ± 19.54%) of u-PA (p>0.999).

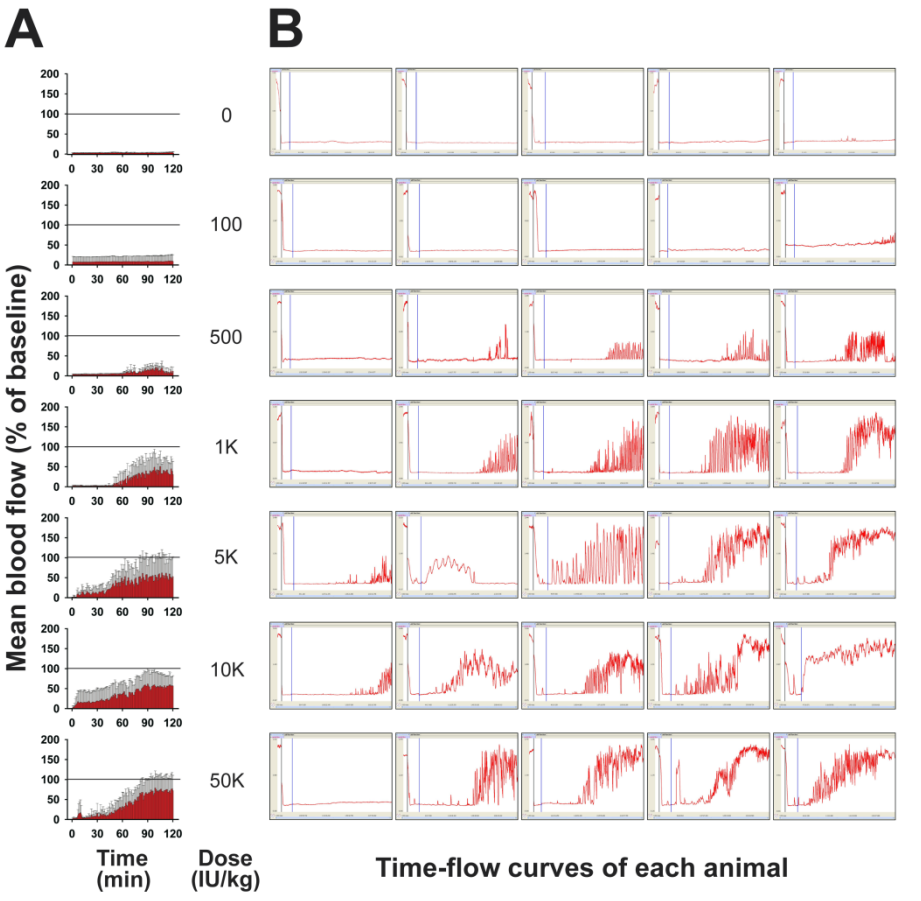

Figure 11. Dose-dependent thrombolytic effect of u-PA. **A:** The mean values of all animals in each group were calculated, and temporal changes are shown as continuous bar graphs (mean ± SD) **B:** Time-flow curves of all mice in each group. Reocclusion occurs relatively frequently during intravenous thrombolytic therapy by u-PA. Time to recanalization and frequency of reocclusion are reduced as the dose of u-PA increases.

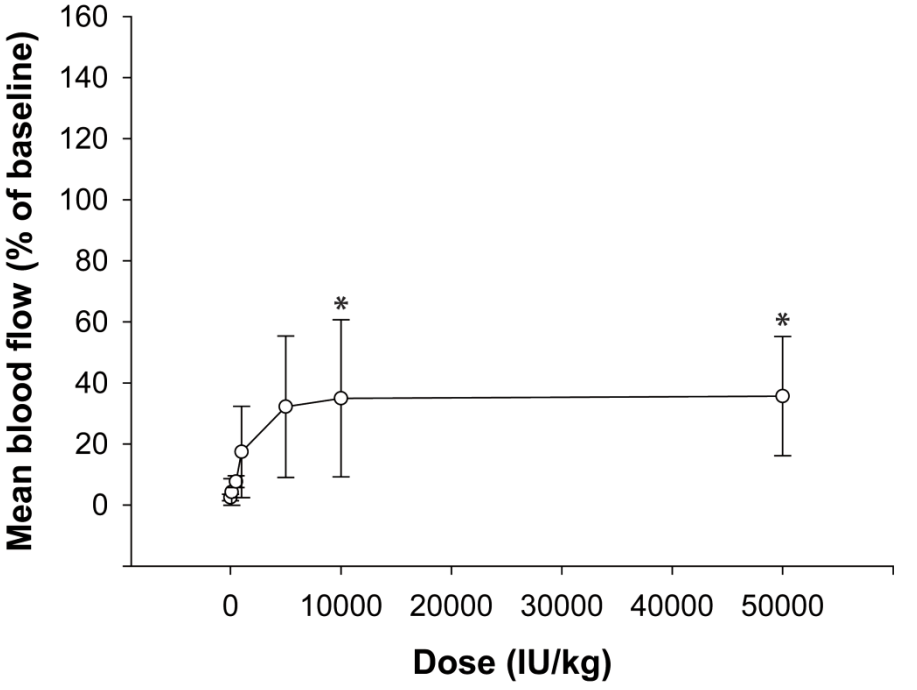

Figure 12. The dose response curve of u-PA. No notable changes are observed until the dosage reaches 500 IU/kg. Restored blood flow is observed at a dose of 1,000 IU/kg, and restoration increases in a dose-dependent manner. Restoration of blood flow is significant at a dose of 10,000 IU/kg ($p=0.049$). There is no significant difference between 10,000 and 50,000 IU/kg of u-PA ($p>0.999$). *$P<0.05$, significantly different from the control normal saline group.

C. Dose-dependent thrombolytic effects of abciximab

Dose-dependent effects of abciximab were determined by calculating the area under the time-flow curve (Figure 13). There were statistically significant differences in thrombolytic effects among the experimental groups ($p<0.001$). Abciximab treatment did not cause any notable change at a dose of 0.25 mg/kg (4.65 ± 1.69%) and 2.5 mg/kg (5.14 ± 2.72%) compared to the status of the normal saline group (2.42 ± 1.07%) (Figure 14). Restored blood flow was observed at a dose of 5 mg/kg (16.32 ± 8.52%), and increased in a dose-dependent manner. The restoration of blood flow was significant at a dose of 20 mg/kg (60.19 ± 42.78%, $p=0.001$). There was no significant difference between 20 and 40 mg/kg (51.45 ± 21.67%) of abciximab ($p=0.992$).

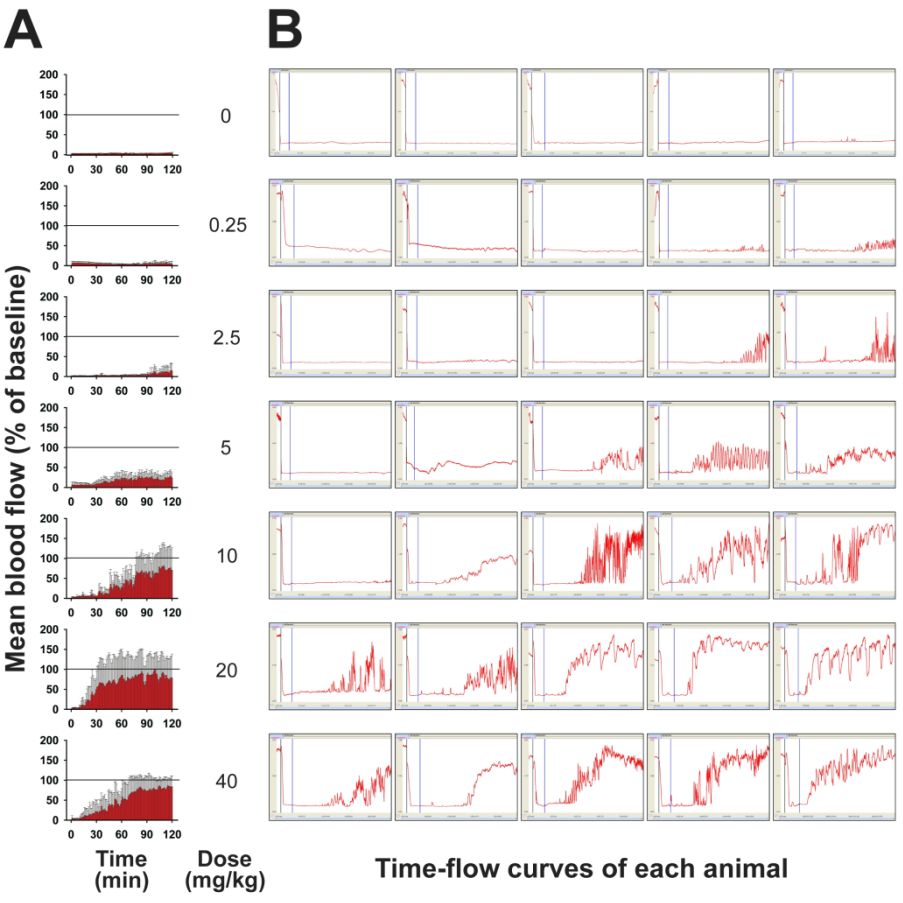

Figure 13. Dose-dependent thrombolytic effect of abciximab. **A:** The mean values of all animals in each group were calculated, and temporal changes are shown as continuous bar graphs (mean ± SD) **B:** Time-flow curves of all mice in each group. Time to recanalization and frequency of reocclusion are reduced as the dose of abciximab increases.

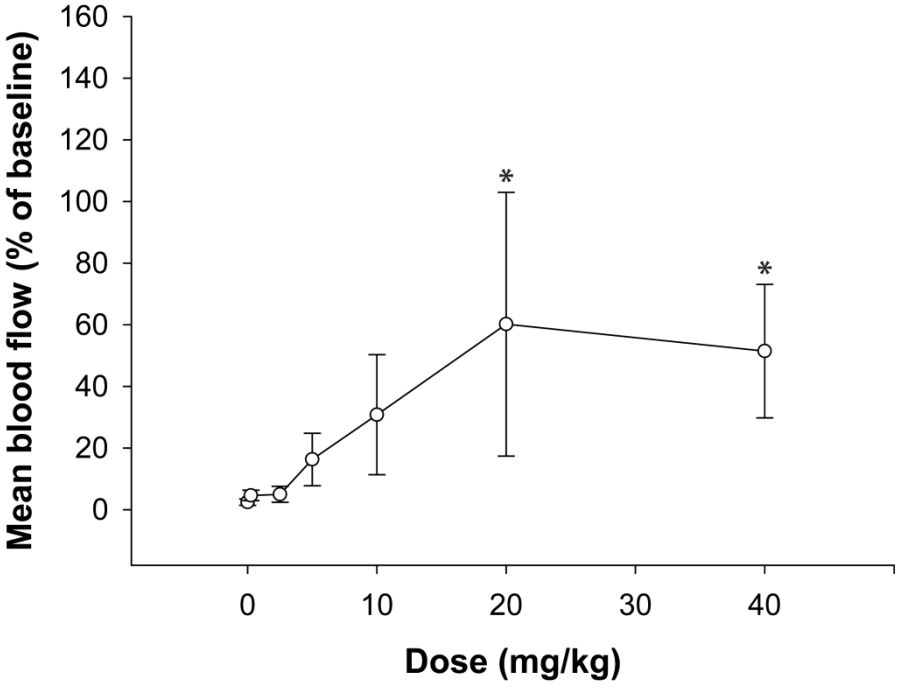

Figure 14. The dose response curve of abciximab. No notable changes are observed until the dosage reaches 2.5 mg/kg. Restored blood flow is observed at a dose of 5 mg/kg, and restoration increases in a dose-dependent manner. Restoration of blood flow is significant at a dose of 20 mg/kg ($p=0.001$). There is no significant difference between 20 and 40 mg/kg of abciximab ($p=0.992$). *$P<0.05$, significantly different from the control normal saline group.

7. The thrombolytic efficacy on the aged thrombus

The blood flow was restored 94.50 ± 20.47% with fresh thrombi, 56.24 ± 38.66% with 1hr-aged thrombi, 17.71 ± 27.27% with 3hr-aged thrombi, 4.48 ± 1.37% with 6hr-aged thrombi, 10.67 ± 6.06% with 12hr-aged thrombi, and 4.51 ± 3.04% with 24hr-aged thrombi by 5 mg/kg of saxatilin (Figure 15). Also, the blood flow was restored 80.52 ± 20.16% with fresh thrombi, 14.26 ± 17.38% with 3hr-aged thrombi, and 6.69 ± 1.66% with 6hr-aged thrombi by 9 mg/kg rt-PA. The thrombolytic efficacy stratified by the age of thrombus (Figure 16).

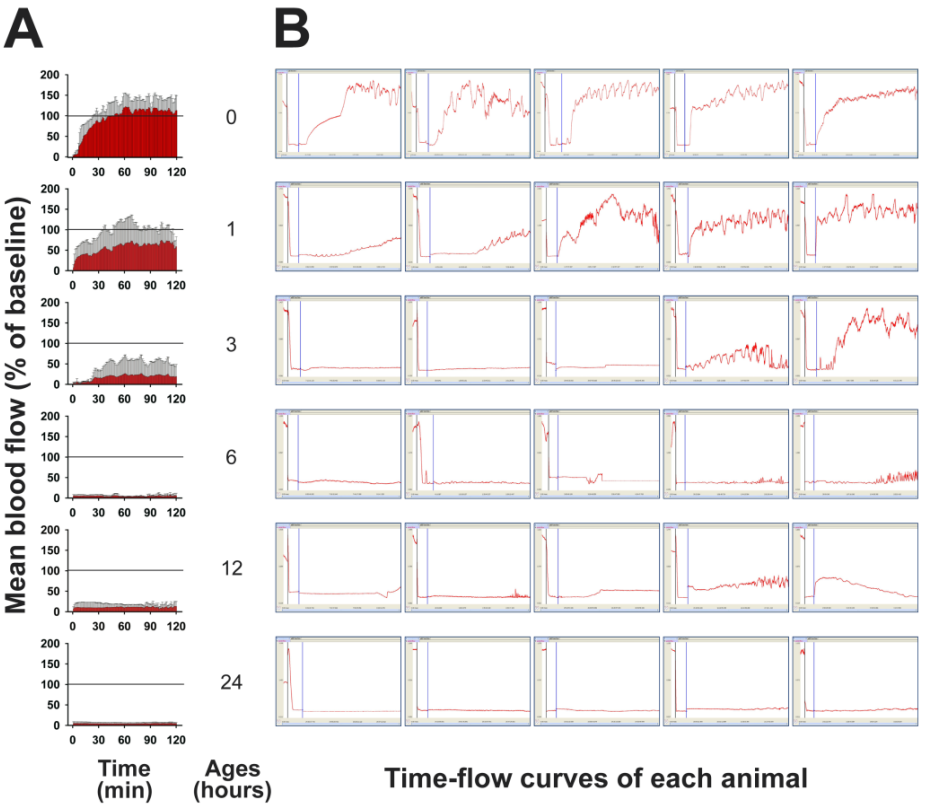

Figure 15. Thrombolytic efficacy of saxatilin on the aged thrombus. **A:** The mean values of all animals in each group were calculated, and temporal changes are shown as continuous bar graphs (mean ± SD) **B:** Time-flow curves of all mice in each group. The effectiveness of thrombolyitic therapy by saxatilin decreases as thrombus age increases.

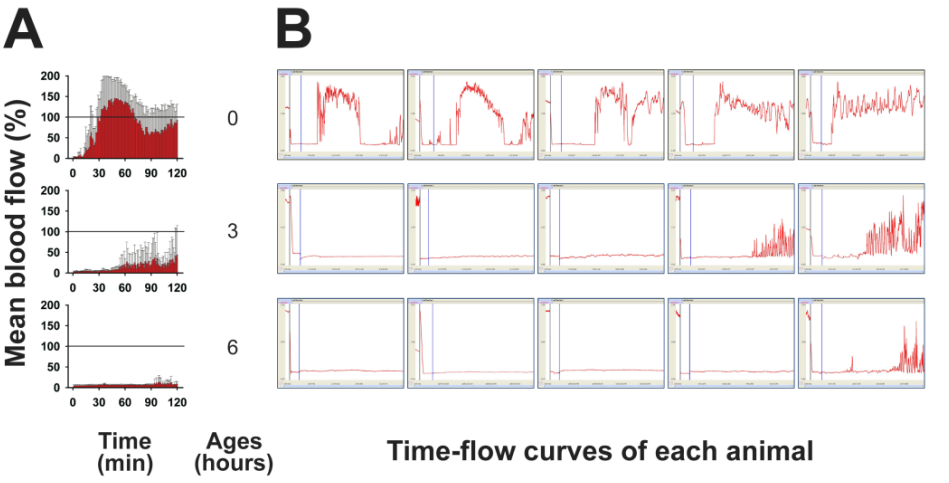

Figure 16. Thrombolytic efficacy of rt-PA on the aged thrombus. **A:** The mean values of all animals in each group were calculated, and temporal changes are shown as continuous bar graphs (mean ± SD) **B:** Time-flow curves of all mice in each group. The effectiveness of thrombolyitic therapy by rt-PA decreases as thrombus age increases.

8. Mortality and bleeding after treatments

A. Saxatilin

Fifty-one animals were used to evaluate the thrombolytic effects of saxatilin. Among them, two animals suffered from bleeding in the cervical incision site, and one of them died of bleeding approximately 90 minutes after the bolus injection of saxatilin. The two animals with bleeding complications were those that received a single bolus dose of 5 mg/kg, and none of mice in the other groups showed bleeding complications.

B. rt-PA

Thirty-one animals were used to evaluate the thrombolytic effects of rt-PA. Among them, two animals suffered from bleeding in the cervical incision site, and one of them died of bleeding approximately 110 minutes after the bolus injection of saxatilin. The two animals with bleeding complications were those that received a dose of 9 mg/kg, and none of mice in the other groups showed bleeding complications.

C. u-PA

Thirty-one animals were used to evaluate the thrombolytic effects of u-PA. Among them, two animals suffered from bleeding in the cervical incision site,

and one of them died of bleeding approximately 80 minutes after the bolus injection of u-PA. The animal died of bleeding complication was those that received a dose of 50,000 IU/kg, the other one with bleeding complication was those that received a dose of 10,000 IU/kg, and none of mice in the other groups showed bleeding complications.

D. Abciximab

None of thirty animals treated with abciximab showed bleeding complications.

9. Effects of saxatilin on platelet aggregation

Platelet aggregometry showed that saxatilin has a strong inhibitory effect on platelet aggregation (Figure 17A). Maximal aggregation values inhibited by each concentration of saxatilin were compared with a control value of maximal aggregation (0 μg/ml). Saxatilin inhibited 50% of the platelet aggregation at a concentration of 0.1 μg/ml (8 ohms), 87.5% at a concentration of 1 μg/ml (7 ohms), and 100% at a concentration of 2 μg/ml (0 ohm) in response to 20 μM of ADP (maximal aggregation of control was 16 ohms) (Figure 17Aa). Also, 63.64% of the platelet aggregation was inhibited at a concentration of 5 μg/ml (8 ohms), 90.91% at a concentration of 50 μg/ml (2 ohms), and 95.45% at a concentration

of 100 μg/ml of saxatilin concentration (one ohm) in response to 5 μg/ml of collagen (maximal aggregation of the control was 22 ohms) (Figure 17Ab).

In experiments using preformed thrombi, the disaggregating effects of saxatilin were dose-dependent (Figure 17B). ADP-induced platelet aggregates were resolved rapidly. At 14 seconds after addition of saxatilin, impedance was restored by 15.79% at a concentration of 10 μg/ml, by 48% at a concentration of 50 μg/ml, and to the basal level (100%) at a concentration of 250 μg/ml (Figure 17Ba). The response to the collagen-induced platelet aggregates was relatively slow. At 300 seconds after saxatilin addition, impedance was restored by 10.32% at a concentration of 10 μg/ml, by 20% at a concentration of 50 μg/ml, and by 31.58% at a concentration of 250 μg/ml (Figure 17Bb). No disaggregating effects were observed when the same volume of normal saline was added.

10. Fibrinolytic effect of saxatilin

Both fibrin and fibrinogen zymograms showed clear bands of rt-PA. However, saxatilin did not demonstrate a clear band of fibrinolysis, which suggests that saxatilin has no fibrinolytic activity (Figure 17C).

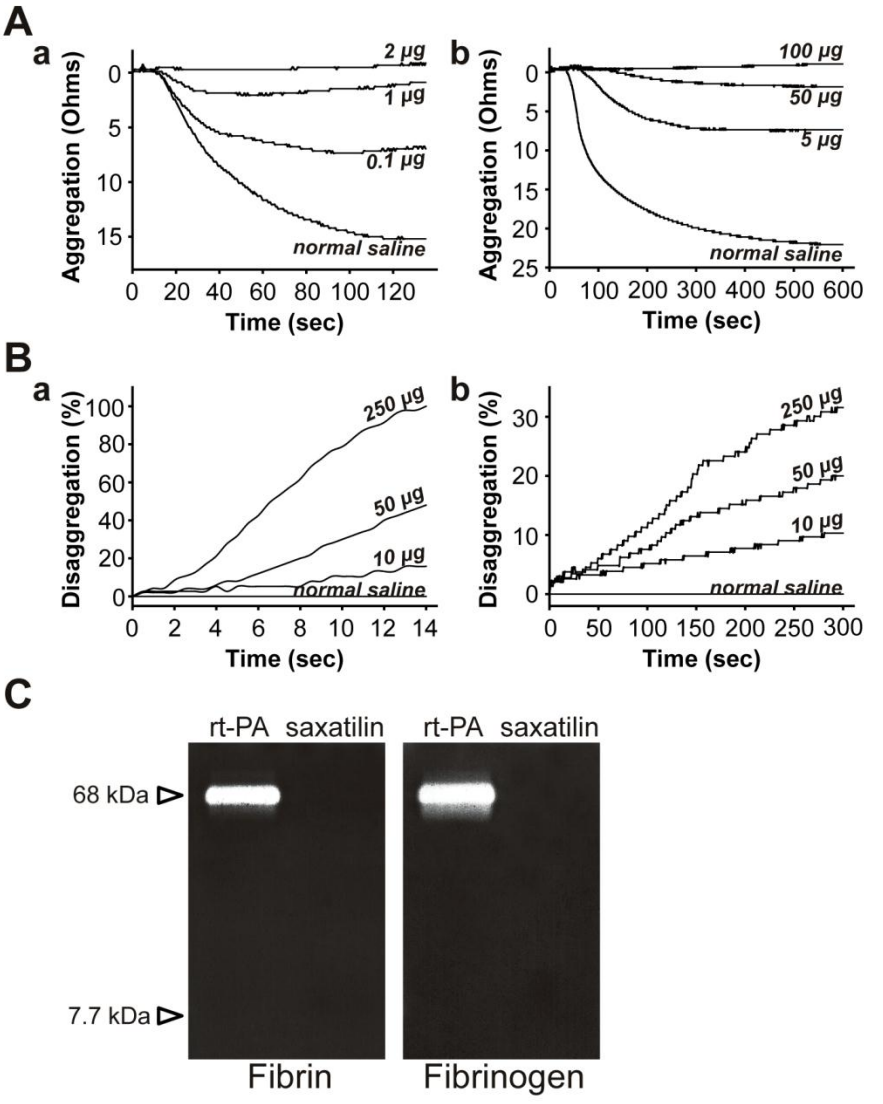

Figure 17. Mechanism of thrombus dissolution by saxatilin. **A:** The inhibitory effect of platelet aggregation by saxatilin. a) The inhibitory effect in response to 20 μM of ADP and b) in response to 5 μg/ml of collagen. Platelet aggregometry shows the dose-dependent inhibitory effect of saxatilin on platelet aggregation. **B:** The effect of saxatilin on platelet disaggregation. a) The effect of saxatilin on the preformed thrombus

induced by 20 μM of ADP, b) induced by 5 μg/ml of collagen. The graphs show dose-dependent disaggregating effect of saxatilin on the preformed thrombus. C: Fibrin/fibrinogen zymography. Both fibrin and fibrinogen zymograms show clear bands of r-tPA. However, a band of fibrinolysis is not seen after saxatilin loading, which suggests that saxatilin has no fibrinolytic activity.

IV. DISCUSSION

Animal models that can effectively evaluate thrombolytic efficacy are indispensible for thrombolytic drug development. In the present study, the thrombolytic effect of saxatilin in mice was evaluated using a $FeCl_3$-induced arterial thrombosis model, which is widely used in various species.[22, 23] Scanning and transmission electron microscopic examinations showed that $FeCl_3$ causes a loss of undulation and severe damage of the endothelium and smooth muscle but does not damage internal elastic lamina, which is consistent with previous observations.[27] The findings of this study suggest that adhesion of platelets to the damaged endothelium and their aggregation initiate platelet-rich thrombi in this model. The size of the thrombus in the model system was also assessed because recanalization induced by thrombolytic agents depends on thrombus size. In this study, thrombus size was consistently similar between mice.

The tools used in this study to measure blood flow are based on ultrasound and platelet aggregometry and are well-established techniques for thrombosis research. However, the assessment methods that were used in this experiment are different from those used in previous studies. In previous studies, the typical

outcome assessment to determine thrombolytic effects was the presence of recanalization. This study, however, assessed and calculated the degree of flow restoration on a minute-to-minute basis using blood flow measurements based on ultrasound. Histological examination showed that the blood flow measurements were a good representation of the degree of recanalization and thrombus resolution. Although recanalization may be the final outcome of thrombolytic drug use, their effects and potency are best tested by degree of thrombus resolution after drug treatment. The assessment method used in this study provided quantitative data for degree of recanalization, time to effective recanalization, and occurrence of reocclusion in real-time. This assessment method may be utilized as a reliable tool for evaluating the in vivo efficacy of new thrombolytic drugs. Thrombolytic effects were also determined using platelet aggregometry. Platelet aggregometry has been used to test inhibition of thrombus formation and thus is typically used for the assessment of platelet antiaggregating drugs. However, this study used platelet aggregometry to assess the platelet disaggregation by administering a thrombolytic drug in preformed thrombi and found that platelet aggregometry can be also used for assessment of thrombolytic effects in vitro. Using this assessment system, the thrombolytic effect of a drug could be examined in a quantitative manner, demonstrating that saxatilin has the potency to dissolve arterial thrombus in vivo and in vitro.

Thrombolytic effects of saxatilin were dose-dependent. Time to recanalization decreased as the dose of saxatilin increased. Reponses to saxatilin varied according to administration methods. When the total dose was administered in a bolus, time to effective recanalization was very fast (approximately three minutes). However, reocclusion was frequently observed, which was also observed in mice treated with a bolus injection of 50% of the total dose. The regimen of a bolus injection of 10% of the total dose and continuous infusion of the remaining dose for one hour resulted in longer time to effective recanalization than that in the groups treated with a high-dose bolus injection. However, this regimen did not show reocclusion patterns. These results may be associated with the relatively short half-life of saxatilin, which was 13.8 minutes in a rat.[25]

The findings of this study indicate that thrombolytic effects of saxatilin work on the platelets rather than on fibrin/fibrinogen. Saxatilin is an RGD-containing protein that inhibits interactions of multiple platelet integrins such as $\alpha_{2b}\beta_3$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ with their ligands. Platelet aggregation is strongly inhibited by disintegrins. RGD-containing proteins from snake venom are characterized by the near absolute conservation of the tripeptide RGD and by the arrangement of disulfide bonds linked by two cysteins. These structural features may play a

critical role in biological activity, with potential as inhibitors for platelet adhesion/aggregation and as antagonists for integrin-mediated adhesion.[28]

In the present study, administration of both 5 mg/kg of saxatilin and 18 mg/kg of rt-PA induced full recanalization (94.50 ± 20.47%, 92.44 ± 27.27%). However, administration of 50,000 IU/kg of u-PA or 20 mg/kg of abciximab, which showed the maximal efficacy in this study, obtained only partial recanalization (35.68 ± 19.54%, 60.19 ± 42.75%). Naturally, direct comparison among these drugs seems to be nonsense because not only do they target different components within the thrombus but they act as via different dethrombotic mechanisms. rt-PA and u-PA are serine protease that catalyzes the conversion of plasminogen to plasmin, resulting in fibrinolysis.[29] On the other hand, abciximab is a human-mouse chimeric antibody which acts as a GP IIb/IIIa antagonist.[10]

Administration of GP IIb/IIIa antagonists not only prevents thrombus formation, but also disaggregates the preformed fresh thrombus through competitive binding to the platelet GP IIb/IIIa receptor against the RGD domain of fibrin or fibrinogen.[10] Saxatilin could lyse the clot by dethrombosis and prevention of rethrombosis, as other GP IIb/IIIa antagonists are known to do.

Considering that abciximab acts by similar dethrombotic mechanisms compared with saxatilin, it was an unexpectable result that abciximab showed low efficacy in this animal model. Small molecular weight drugs have a higher disaggregation potential than large molecular weight drugs.[10] The smaller molecular weight (7.7 kDa) of saxatilin might also play a beneficial role in more effective thrombus dissolution than the larger molecular weight (47.6 kDa) of abciximab. Saxatilin may have additive effects on dethrombosis and antithrombosis by acting against multiple integrins. In addition to $\alpha_{2b}\beta_3$, other integrins such as $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ also mediate platelet function.[30] Inhibition of GP IIb/IIIa does not produce complete platelet adhesion/aggregation, but a multiple integrin-ligand association synergizes during platelet adhesion and aggregation.[31] Firm platelet adhesion, which is followed by an initial interaction between GP1b and the von Willebrand factor, is mediated by integrins such as $\alpha_5\beta_1$, $\alpha_1\beta_1$, and $\alpha_{2b}\beta_3$.[32] Integrins $\alpha_5\beta_1$ and $\alpha_{2b}\beta_3$ share the binding site of fibrinogen to platelets.[33] Integrin $\alpha_5\beta_1$ plays a role in contraction of fibrin clots as well as platelet adhesion.[34] Integrin $\alpha_v\beta_3$ also plays a role in thrombus formation by adhering not only to ligands for $\alpha_{2b}\beta_3$ (i.e., von Willebrand factor, fibrinogen, fibronectin, vitronectin, and thrombospondin), but also to collagen.[32, 35] In this regard, maximal disaggregation of platelets from fibrinogen as well as inhibition of platelet adhesion/aggregation and thrombus growth may be

achieved by blocking the actions mediated by multiple integrins on platelets.

Thrombolytic efficacy was directly related to the thrombus age. The effectiveness of thrombolyitic therapy by not only saxatilin also rt-PA decreased as thrombus age increased. It is considered as a general feature of thrombolytic agents on the aged thrombus.

Saxatilin seems to be safe in that the effective dose is relatively low and it rarely causes bleeding complications. The most efficient dose in this study (5 mg/kg) was much lower than the known LD_{50} of saxatilin (400 mg/kg) in ICR mice.[25] Among 51 mice that were administered saxatilin in this study, only two mice bled at the cervical incision site. Moreover, these two mice received a single bolus dose. Therefore it appears that 5 mg/kg of saxatilin, which is the most effective dose, is also a safe dose.

V. CONCLUSION

Disaggregation of platelets from fibrin is a potential approach to dissolving thrombi.[10, 11] Several specific GP IIb/IIIa receptor inhibitors have been developed and are currently available in clinics. However, saxatilin, which originates from natural sources and inhibits multiple integrins acting on platelets, may be a good candidate for a new thrombolytic drug with improved potency.

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ABSTRACT (In Korean)

새로운 혈전용해제 후보물질로서 삭사틸린의 혈전용해효과

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권 일

한국 까치살모사(*Gloydius saxatilis*)의 독에서 정제 후 복제한 새로운 디스인테그린인 삭사틸린(saxatilin)은 혈소판의 $\alpha_{2b}\beta_3$ (GP IIb/IIIa), $\alpha_5\beta_1$, 또는 $\alpha_v\beta_3$ 등의 인테그린과 결합하여 혈소판의 활성화와 응집을 강력하게 억제하는 작용을 한다고 알려져 있다. 하지만, 삭사틸린이 이미 형성되어있는 혈전을 용해하는지의 여부는 현재까지 전혀 밝혀진 바가 없다. 따라서 본 연구에서는 염화제이철($FeCl_3$)에 의해 유도

되는 마우스 동맥 내 혈전생성 모델을 이용하여 삭사틸린의 혈전용해 효과를 검증하였다. 혈전생성에 의한 동맥의 폐색과 혈전용해치료에 의한 동맥의 재관통 정도는 초음파 혈류량계를 이용하여 실시간으로 혈류량을 측정함으로써 평가했고, 형태학적 관찰을 통해 재검증하였다. 또한, 상용화된 혈전용해제인 rt-PA와 u-PA, abciximab의 혈전용해효과를 평가하고, 각각의 용해패턴을 삭사틸린과 비교 분석하였다. 추가로, 생성 후 경과된 시간이 각각 다른 혈전에 대한 삭사틸린의 용해효과를 검증했고, 동일한 시간이 경과된 혈전에 대한 rt-PA의 효과를 삭사틸린과 비교하였다. 연구 결과, 삭사틸린은 투여용량에 의존적인 혈전용해효과를 보였다. 2.5 mg/kg 용량의 삭사틸린을 투여했을 때부터 혈전용해효과가 관찰되어, 3.75 mg/kg 용량을 투여했을 때 통계적으로 의미 있는 효과를 보였으며, 5 mg/kg 용량을 투여했을 때 거의 정상수준으로 혈류량을 회복하였다. 또한, 삭사틸린의 투여용량을 높일수록 유효 재관통(effective recanalization)에 이르는 소요시간을 단축할 수 있었다. 삭사틸린의 투여방법에 따른 혈전용해효과를 비교했을 때, 전체 용량의 10%를 볼루스로 투여하고 나머지 용량을 한 시

간 동안 연속투여하는 방법에서 재폐색 없이 가장 효과적으로 재관통되는 것을 확인하였다. rt-PA, u-PA, 그리고 abciximab과 비교했을 때, 삭사틸린은 더욱 강력한 혈전용해효과를 보임과 더불어 폐색된 혈관이 재관통되기까지 소요되는 시간을 현저하게 줄였으며, 재관통 이후 재폐색이 거의 없이 혈류를 안정적으로 유지시키는 것을 확인하였다. 생성 후 시간이 경과된 혈전에서, 경과시간이 길수록 삭사틸린과 rt-PA의 혈전용해효과가 모두 감소함을 확인하였다. 삭사틸린을 투여한 51 마리의 마우스 중에서 5 mg/kg 전체용량을 볼루스로 투여한 두 마리에서만 경부의 절개부위에서 약간의 출혈이 관찰되었을 뿐, 나머지 동물에서는 출혈이 관찰되지 않았다. 삭사틸린의 혈전용해기전을 규명하기 위해 수행한 피브린/피브리노겐 자이모그래피(zymography)와 혈소판 응집시험(platelet aggregometry)에서 삭사틸린은 혈전 내의 피브린 또는 피브리노겐을 분해하는 기전이 아닌, 혈소판의 다양한 인테그린에 대해 피브린과 경쟁적으로 결합함으로써 혈전을 용해하고 재폐색을 억제한다는 것을 확인하였다. 본 연구에서 이용한 염화제이철에 의해 유도되는 동맥 내 혈전생성 모델에서, 혈전을 효과적으로

용해할 수 있는 용량의 삭사틸린을 투여해도 출혈의 위험이 없이 안전함을 확인하였다. 혈전용해치료에 있어서 피브린과 결합하거나 서로 응집하여 혈전을 구성하는 혈소판을 분해하는 치료가 유용할 것으로 여겨지며, 실제 임상에서도 혈소판의 GP IIb/IIIa 수용체를 타겟으로 하는 약제들이 개발되어 사용되고 있다. 본 연구는, 천연물에서 유래하였으며 혈소판의 다양한 인테그린에 결합하여 혈소판의 활성화와 응집을 억제하는 기전의 삭사틸린이, 기존의 상용화된 혈전용해제에 비해 더욱 뛰어난 효능의 새로운 혈전용해제로의 발전 가능성이 있음을 시사하는 바이다.

핵심되는 말: 삭사틸린, 염화제이철에 의해 유도되는 동맥 내 혈전생성, 혈전용해치료, 항응집수용체 치료

PUBLICATION LIST

1. Kwon I, Kim EH, del Zoppo GJ, Heo JH. Ultrastructural and temporal changes of the microvascular basement membrane and astrocyte interface following focal cerebral ischemia. J Neurosci Res 2009;87(3):668-76.

PATENT LIST

1. **10-2010-0091500** *Methods for Real-Time Measurement of Thrombi in a Blood Vessel Using Doppler*

2. **10-2010-0107760** *Composition for Thrombolysis and Pharmaceutical Composition for Treating Diseases related to Blood Vessel Occlusion or Narrowness Comprising the Same*

3. **10-2011-0112953** *Composition for Thrombolysis and Pharmaceutical Composition for Treating Diseases related to Blood Vessel Occlusion or Narrowness Comprising the Same*