

Original Article

Plasminogen potentiates thrombin cytotoxicity and contributes to pathology of intracerebral hemorrhage in rats

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Running headline; Plasminogen in hemorrhagic brain injury

## **Abstract**

Thrombin and plasmin are serine proteases involved in blood coagulation and fibrinolysis, whose precursors are circulating in blood stream. These blood-derived proteases may play important roles in the pathogenesis of intracerebral hemorrhage by acting on brain parenchymal cells. We previously reported that thrombin induced delayed neuronal injury through extracellular signal regulated kinase (ERK)-dependent pathways. Here we investigated potential cytotoxic actions of plasminogen, a precursor protein of plasmin, using slice cultures prepared from neonatal rat brain and intracortical microinjection model in adult rats. Although plasminogen alone did not evoke prominent neuronal injury, plasminogen caused significant neuronal injury when combined with a moderate concentration of thrombin (30 U/ml) in the cerebral cortex of slice cultures. The cortical injury was prevented by tranexamic acid and aprotinin. The combined neurotoxicity of thrombin and plasminogen was also prevented by PD98059, an inhibitor of ERK pathway, as well as by other agents that have been shown to prevent cortical injury induced by a higher concentration (100 U/ml) of thrombin alone. ERK phosphorylation after plasminogen exposure was localized in cortical astrocytes. Moreover, microinjection of plasminogen *in vivo* potentiated thrombin-induced cortical injury, and inhibition of plasmin ameliorated hemorrhage-induced neuronal loss in the cerebral cortex. These results suggest that plasminogen/plasmin system augmenting thrombin neurotoxicity participates in hemorrhagic cortical injury.

Keywords: collagenase; cortical cells; hemorrhagic brain injury; plasminogen; slice cultures; thrombin

## Introduction

Intracerebral hemorrhage (ICH) represents an acute stroke characterized by extravasation of blood into brain parenchyma and formation of hematoma. While ICH accounts for 15% of all strokes, no satisfactory medical treatments have been developed against this disorder, and patients suffer from poor prognosis and high mortality. After hemorrhagic ictus, hematoma may cause brain injury via cytotoxicity of blood-derived molecules as well as via mass effects (Xi *et al.*, 2006). Thrombin is one of the major blood-derived serine proteases released into brain parenchyma when cerebral vessels are ruptured. Besides its important role in blood coagulation, thrombin can induce neuronal apoptotic death and microglial activation in various experimental models (Donovan *et al.*, 1997; Fujimoto *et al.*, 2006, 2007).

Plasmin is another serine protease derived from a circulating precursor protein, plasminogen. Cleavage between Arg-560 and Val-561 of plasminogen molecule, which is catalyzed by plasminogen activators such as urokinase and tissue-type plasminogen activators, generates plasmin (Robbins *et al.*, 1967). Plasmin is well known as a fibrinolytic serine protease that interacts with polymerized fibrin clots to release free fibrin molecule and dissolve clots (Castellino and Ploplis, 2005). The catalytic domain of plasmin is localized in the light chain derived from the carboxyl-terminus of plasminogen, whereas five kringle domains are within the heavy chain from the amino-terminus of plasminogen (Patthy, 1985). The kringle domain of plasmin(ogen) possesses lysine-binding activity, and plasminogen binds its substrates such as fibrin through lysine-binding sites, which results in the enhancement of plasmin generation (Hajjar *et al.*, 1986; Wu *et al.*, 1990).

Plasmin(ogen) may have physiological/pathophysiological functions in the central nervous

system, since expression of plasminogen has been detected in central neurons (Tsirka *et al.*, 1997a). In a pathological context, neurotoxicity of plasminogen in the striatum has been demonstrated in an *in vivo* experimental model (Xue and Del Bigio, 2001, 2005). In addition, plasmin-mediated degradation of the extracellular matrix enhances vulnerability of hippocampal neurons to excitotoxins (Chen and Strickland, 1997; Tsirka *et al.*, 1997b). A detrimental role of plasminogen/plasmin system in ischemic brain injury has also been proposed (Takahashi *et al.*, 1997).

When ICH occurs, circulating plasminogen and plasminogen activators are released into the cerebral parenchyma, and plasmin generated at the hemorrhagic sites may contribute to the brain injury after ICH. In the present study, we investigated pathogenic roles of plasminogen with relation to ICH. We particularly focused on the interaction of plasminogen with the neurotoxic actions of thrombin.

## **Materials and Methods**

### *Drugs and chemicals*

Drugs and chemicals were obtained from Nacalai Tesque (Kyoto, Japan), unless otherwise indicated. Thrombin from bovine plasma (catalog No. T4648), bovine serum albumin (catalog No. A2153), aprotinin from bovine lung and clodronate were obtained from Sigma (St. Louis, MO, USA). Tranexamic acid was from Aldrich (St. Louis, MO, USA).

Plasminogen from human plasma (catalog No. 528175), plasmin from human plasma (catalog No. 527621), PD98059, SB203580,

4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) and

bisindolylmaleimide (BIM) were from Calbiochem (San Diego, CA, USA). SP600125 was

from Tocris Cookson (Bristol, UK). Argatroban was from Sawai Pharmaceutical (Osaka,

Japan). Cycloheximide was from Wako Pure Chemical (Osaka, Japan).

### *Preparation of slice cultures*

All experimental procedures were approved by our institutional animal experimentation committee, and animals were treated in accordance with the guidelines of the NIH regarding the care and use of animals for experimental procedures. Organotypic slice cultures were prepared essentially according to the methods described previously (Fujimoto *et al.*, 2006).

Coronal brain slices of 300  $\mu$ m thickness were prepared from Wistar rats at postnatal days 3 - 4 (Nihon SLC, Shizuoka, Japan), and six cortico-striatal tissue slices were transferred onto a 30-mm Millicell-CM insert membrane (Millipore, Bedford, MA, USA) in six-well plates.

Culture medium, consisting of 50% minimum essential medium/HEPES (GIBCO, Invitrogen Japan, Tokyo, Japan), 25% Hanks' balanced salt solution (GIBCO) and 25% heat-inactivated

horse serum (GIBCO) supplemented with 6.5 mg/ml D-glucose and 2 mM L-glutamine, 100 U/ml penicillin G potassium and 100 µg/ml streptomycin sulfate (GIBCO), was supplied at 0.75 ml/well so that the slices were maintained at liquid/air interface. Culture medium was replaced with fresh one on the next day of culture preparation, and thereafter, every two days. Slices were cultured in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 34°C.

#### *Drug treatment and cell death assessment*

Cultured slices at 9 - 11 days *in vitro* were incubated for 24 - 48 h in serum-free medium, where minimum essential medium/HEPES substituted for horse serum. Then slices were exposed to drugs and chemicals dissolved in serum-free medium for indicated periods. To assess cell injury, propidium iodide (PI; 5 µg/ml, Wako Pure Chemical) was added to serum-free medium for drug treatment. After 72 h, images of PI fluorescence of each slice were captured through a monochrome chilled CCD camera (C5985; Hamamatsu Photonics, Hamamatsu, Japan) and stored as image files. The average signal intensity in an area of 180 µm × 180 µm within the parietal cortex was obtained as the fluorescence value of each slice, with the use of NIH Image 1.63 software. In each experiment, slice cultures treated with 100 µM *N*-methyl-D-aspartate (NMDA) for 72 h were used to determine the degree of the standard injury. Fluorescence values were normalized with the intensity of cultures that received standard injury as 100%. Images of whole slice cultures were also obtained, and the area of the striatal region in each slice was estimated with NIH Image 1.63.

#### *Immunohistochemistry for slice cultures*

After drug treatment, slice cultures were fixed with 0.1 M phosphate buffer containing 4%

paraformaldehyde and 4% sucrose for 2 h. After rinsing with phosphate-buffered saline (PBS), they were permeabilized and blocked with 0.2% Triton X-100 in PBS containing 1.5 % goat serum followed by incubation with primary antibodies overnight at 4°C. Primary antibodies were mouse anti-NeuN (1:200, Chemicon International, Temecula, CA, USA), mouse anti-OX42 (1:300, Dainippon Pharmaceutical, Osaka, Japan), mouse anti-glia fibrillary acidic protein (GFAP) (1:500, Sigma) and rabbit anti-phospho-p44/42 MAP kinase (T202/Y204) (1:250, Cell Signaling Technology, Beverly, MA, USA). After rinse with PBS, cultures were incubated with secondary antibodies for 1 h at room temperature. Alexa Fluor 488-labeled goat anti-mouse IgG (1:200, Molecular Probes, Eugene, OR, USA), Alexa Fluor 594-labeled goat anti-mouse IgG (1:200, Molecular Probes) and Alexa Fluor 488-labeled goat anti-rabbit IgG (1:200, Molecular Probes) were used as secondary antibodies. Then cultures were rinsed with PBS and specimens were dehydrated through a graded ethanol series and mounted on slide glasses with glycerol. Fluorescence signals were observed with a laser-scanning confocal microscopic system (MRC1024, Biorad, Hercules, CA, USA).

#### *Plasmin activity quantification*

Protease activity of plasmin was measured by the rate of substrate cleavage according to a previous report (Schneider and Nesheim, 2004) with modification. D-Val-Leu-Lys *p*-nitroanilide (S2251 reagent, Sigma), a chromogenic substrate of plasmin, was dissolved in 50 mM Tris-buffered saline at a concentration of 2 mM. Serum-free media with or without drugs and culture supernatants were reacted with an equal amount of S2251 solution at room temperature. Absorbance of *p*-nitroaniline generated by the cleavage of S2251 reagent was measured at 450 nm on a microplate reader from 3 min to 180 min after initiation of the

reaction.

### *Western blot analysis*

After treatment with drugs for indicated periods, slice cultures were harvested and homogenized in ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.0), 25 mM  $\beta$ -glycerophosphate (Sigma), 2 mM EGTA·2Na, 1% Triton X-100, 1 mM vanadate, 1% aprotinin (Sigma), 1 mM phenylmethylsulfonyl fluoride and 2 mM dithiothreitol. Samples were mixed with sample buffer composed of 124 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 10% glycerol, 0.02% bromophenol blue and 4% 2-mercaptoethanol. After boiling for 5 min, samples were subjected to 12% SDS-polyacrylamide gel electrophoresis for 70 min, followed by transfer to PVDF membrane (Millipore) for 70 min. Membranes were blocked with 5% nonfat milk and subsequently probed overnight with mouse anti-phospho-p44/42 MAP kinase (T202/Y204) (1:2000, Cell Signaling Technology) and anti-p44/42 MAP kinase (1:1000, Cell Signaling Technology). The membranes were rinsed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:10000, Jackson Immunoresearch Laboratories, West Grove, PA, USA) or goat anti-rabbit IgG (1:10000, Jackson Immunoresearch Laboratories). Following incubation with secondary antibodies, membranes were rinsed and bound antibodies were detected with enhanced chemiluminescence kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. The band intensities were analyzed with NIH image 1.63.



### *Surgical procedures for microinjection and drug administration*

Male Sprague-Dawley rats initially weighing 220 - 280 g were used. Animals were kept at constant ambient temperature ( $23 \pm 2$  °C) under a 12-h light and dark cycle with free access to food and water. Drug administration was performed according to a previous report (Fujimoto *et al.*, 2007) with modification. Briefly, rats were anesthetized with pentobarbital (50 mg/kg, i.p., Dainippon Sumitomo Pharmaceutical, Osaka, Japan) and placed in a stereotaxic frame (Narishige, Tokyo, Japan). After scalp incision, a hole was drilled on the skull. Thrombin, plasminogen and collagenase were administered via a stainless steel injection cannula (o.d. 0.35 mm) into the parietal cortex at 0.2 mm anterior, 3.0 mm lateral, 2.5 mm ventral from bregma. The solution of these drugs in a volume of 2  $\mu$ l/rat was infused at a constant rate of 0.5  $\mu$ l/min. The injection cannula was left in place at least for additional 5 min to prevent backflow of drugs. Aprotinin and tranexamic acid were given intraperitoneally. Aprotinin was administered at a dose of 2 mg/kg at 30 min after intracortical injection of collagenase, followed by administration of 1 mg/kg from the next day twice daily. Tranexamic acid was administered at a dose of 300 mg/kg at 30 min after intracortical injection of collagenase, followed by administration of 150 mg/kg from the next day twice daily. Control rats received intraperitoneal injection of the equivalent volume of saline.

### *Histological examinations after microinjection*

After indicated periods from intracortical injection, rats were anesthetized again and perfused through the heart with PBS followed by 4% paraformaldehyde. Brains were removed from the skull, and postfixed in 4% paraformaldehyde and dehydrated with 15%

sucrose solution for overnight at 4°C. After freezing, coronal brain sections (16 µm) containing the injection site were prepared and mounted onto slides. For immunohistochemistry, specimens were autoclaved (121°C for 15 min) for epitope retrieval. They were permeabilized and blocked with 0.5% Triton X-100 in PBS containing 1.5% horse serum for 1 h at room temperature. Specimens were then incubated with mouse anti-NeuN (1:200) overnight at 4°C, and after rinse with PBS, they were incubated with biotinylated horse anti-mouse IgG (1:200, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. After rinse, specimens were treated with avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories), and then peroxidase was visualized with diaminobenzidine and H<sub>2</sub>O<sub>2</sub>. Bright-field images were captured through a monochrome chilled CCD camera. For injury induced by thrombin with or without plasminogen, we defined the region where no NeuN-positive cells were observed as the injured region, and measured the injured area with NIH Image 1.63. For injury resulting from collagenase-induced hemorrhage, we counted the number of NeuN-positive cells in an area of 230 µm × 320 µm at the center of hematoma formed in the cortex.

### *Statistics*

Data are expressed as means ± SEM. Statistical significance of difference was evaluated with paired *t*-test, Mann-Whitney U-test or one-way analysis of variance followed by Student–Newman–Keuls' test. Probability values less than 5% were considered significant.

## Results

We applied plasminogen (0.1 – 10 U/ml) for 72 h to cortico-striatal slice cultures. PI fluorescence was used as a measure of cell injury in the cortical region. Plasminogen up to 10 U/ml did not induce cortical cell injury (Fig. 1F). Moreover, while we have previously shown that thrombin induced shrinkage of the striatal tissue in slice culture (Fujimoto *et al.*, 2006), we did not observe any changes in the striatal area after application of plasminogen (data not shown). Notably, when plasminogen at 3 U/ml, but not at 1 U/ml, was applied to slice cultures concomitantly with a moderate concentration (30 U/ml) of thrombin that did not induce prominent cortical injury by itself, a significant degree of cortical injury was evident at 72 h (Fig. 1A-D, G). When plasminogen was applied from 24 h before application of 30 U/ml thrombin, significant cortical injury was produced also at 1 U/ml and in a concentration-dependent manner between 1 – 3 U/ml (data not shown). PI fluorescence in the cortical region of slice cultures treated with a combination of thrombin and plasminogen was colocalized with immunoreactivity against NeuN, a neuronal marker (Fig. 1E), suggesting that neurons were injured by this treatment. On the other hand, plasminogen did not potentiate NMDA-induced cortical neuron injury, even when slices received 24-h pretreatment with plasminogen (Fig. 1H). In addition, plasminogen did not affect the degree of striatal shrinkage induced by thrombin (Fig. 1I). Plasminogen did not show any toxic effects when combined with bovine serum albumin at a protein concentration corresponding to that of 30 U/ml thrombin (data not shown). These results suggest that plasminogen specifically evokes cortical injury by a combination with thrombin.

Plasminogen can be converted into a serine protease plasmin by plasminogen activators. We then examined the involvement of plasmin protease activity on cortical injury induced by

thrombin and plasminogen. To measure plasmin activity, we incubated serum-free medium containing plasmin (0.1 U/ml) or plasminogen (3 U/ml) with a chromogenic substrate, S2251, at room temperature. Analysis of the absorbance of the degradation product indicated that plasmin cleaved S2251 time-dependently, whereas medium alone or plasminogen was without effect (Fig. 2A), confirming that S2251 was a suitable substrate of plasmin but not of plasminogen. Based on these observations, we examined whether plasmin was generated after application of plasminogen to slice cultures. The supernatants of slice cultures treated with 3 U/ml plasminogen for 72 h cleaved S2251 gradually, whereas those of slice cultures treated with vehicle showed no effect (Fig. 2B). These results suggest that a fraction of plasminogen was converted into plasmin during incubation with slice cultures. Moreover, cortical injury induced by thrombin plus plasminogen was prevented by aprotinin (20 µg/ml), a plasmin inhibitor, and tranexamic acid (3 mM), a lysine analog that inhibits plasminogen activation (Fig. 2C, D). In this set of experiments, we used aprotinin and tranexamic acid at concentrations that were sufficient to inhibit plasmin activity but did not inhibit thrombin activity (Engles, 2005). Indeed, aprotinin and tranexamic acid did not ameliorate cortical injury induced by a high concentration (100 U/ml) of thrombin (Fig. 2E, F), suggesting that the effects of these drugs were mediated by inhibition of plasmin activity.

We previously demonstrated that thrombin-induced cortical injury was prevented by inhibition of extracellular signal-regulated kinase (ERK) pathway, Src family tyrosine kinase, protein kinase C (PKC) and *de novo* protein synthesis as well as by an inhibitor of thrombin protease activity. In contrast, inhibition of p38 mitogen-activated protein kinase (MAPK) and depletion of microglia had no effect, and inhibition of c-Jun N-terminal kinase (JNK) exacerbated thrombin-induced cortical injury (Fujimoto *et al.*, 2006). Then, we examined

the involvement of these pathways in neurotoxicity of thrombin plus plasminogen. Selective inhibitors for various signaling pathways were used at the same concentrations as those used in our previous study (Fujimoto et al., 2006). As shown in Fig. 3A-E, the combined neurotoxicity of thrombin and plasminogen in the cortical region was prevented by inhibitors of ERK pathway (PD98059, 100  $\mu$ M), Src family tyrosine kinase (PP2, 100  $\mu$ M), PKC (BIM, 3  $\mu$ M), *de novo* protein synthesis (cycloheximide, 1  $\mu$ g/ml) and thrombin protease activity (argatroban, 300  $\mu$ M). On the other hand, an inhibitor of p38 MAPK (SB203580, 100  $\mu$ M) and depletion of microglia by clodronate (100  $\mu$ g/ml) did not prevent cortical injury, and an inhibitor of JNK (SP600125, 100  $\mu$ M) exacerbated the injury (Fig. 3F-H). Overall, the effects of various agents on neurotoxicity of thrombin plus plasminogen were similar to those on neurotoxicity of a high concentration of thrombin alone (Fujimoto, *et al.*, 2006).

We further investigated the involvement of ERK in combined neurotoxicity of thrombin and plasminogen. Levels of ERK phosphorylation in slice cultures were examined by Western blot analysis after 3 h or 24 h of incubation with or without thrombin and plasminogen. At 3 h, plasminogen (3 U/ml) increased phosphorylated ERK level to  $267 \pm 55\%$  of vehicle treatment ( $n=8$ ,  $P=0.019$ , evaluated by paired *t*-test). The elevated level of ERK phosphorylation was sustained even after 24 h from the onset of plasminogen treatment (Fig. 4A). Consistent with our previous study (Fujimoto *et al.*, 2006), thrombin also caused ERK phosphorylation at 3 h, which declined thereafter (Fig. 4A). To determine cell types exhibiting ERK phosphorylation after 3 h of plasminogen treatment, we performed immunofluorescence staining with a combination of antibodies against phosphorylated ERK and cell type-specific marker proteins. The majority of phosphorylated ERK immunofluorescence in the cortical region was colocalized with immunofluorescence of an

astrocyte marker, GFAP (Fig. 4B), but not with a neuronal marker, NeuN (Fig. 4C) or a microglial marker, OX42 (data not shown). These results suggest that plasminogen evokes persistent ERK phosphorylation in cortical astrocytes.

On the basis of these *in vitro* observations, we next examined whether plasminogen could potentiate thrombin neurotoxicity *in vivo*. For this purpose, we injected thrombin with or without plasminogen into the rat cortex. The dose of thrombin was set to 1 U, because in preliminary experiments 10 U thrombin alone caused severe cortical injury. After 72 h, several coronal brain sections were prepared from each rat and immunostained with anti-NeuN antibodies. As shown in Fig. 5A, thrombin injection into the cerebral cortex produced a distinct region with decreased NeuN immunoreactivity around the injection site, as with the case of thrombin injection into the striatum in our previous study (Fujimoto *et al.*, 2007). Since there were very few NeuN-positive cells within this region and we could easily distinguish the border between the injured region and the intact region, we assessed the degree of neurotoxicity by the injured area defined as the region with scarce NeuN-immunoreactivity around the injection site. As expected, the injured area of the section containing the injection site, as revealed by a cannula track, was the largest among several coronal brain sections examined. This means that the cortical injury expanded concentrically from the injection site. In this series of experiments, plasminogen (0.3 U) again augmented thrombin-induced cortical injury at 72 h (Fig. 5B and C).

Finally, we examined possible involvement of plasminogen in hemorrhage-induced brain injury *in vivo*. Injection of 0.1 U collagenase into the rat cortex led to hematoma formation and a substantial decrease of NeuN-positive cells inside the hematoma. Unlike in the case of thrombin injection, however, a moderate number of NeuN-positive cells remained viable even

in the center of the collagenase injection site (Fig. 6A, B, E, F). Therefore, we evaluated neuroprotective effects of drugs by the number of NeuN-positive cells at the hematoma center 72 h after collagenase injection. Aprotinin (2 mg/kg/day) intraperitoneally administered after collagenase injection partially but significantly increased the number of NeuN-positive cells at the hematoma center, compared with saline-administered control (Fig. 6C, G, I). Tranexamic acid (300 mg/kg/day) also tended to increase the number of surviving NeuN-positive cells, although the effect did not reach statistical significance (Fig. 6D, H, I). The dose of aprotinin in this set of experiments was almost maximal one that could be prepared from the commercial product (Sigma, A6279), and the dose of tranexamic acid was chosen according to a previous report (O'Brien et al., 2000). We also compared the hematoma size of each treatment group. When the area of hematoma was defined as the cortical region with decreased NeuN-immunoreactivity in the coronal brain section containing the injection site, hematoma size of saline-administered control was  $2.12 \pm 0.28 \text{ mm}^2$  (n=10). Hematoma sizes of rats treated with aprotinin and tranexamic acid were  $2.63 \pm 0.30 \text{ mm}^2$  (n=9) and  $2.70 \pm 0.41 \text{ mm}^2$  (n=7), respectively, both of which were not significantly different from that of control group. These results suggest that neuroprotective effect afforded by regulation of plasmin activity is independent of inhibition of fibrinolytic activity of plasmin.

## Discussion

Plasminogen is a circulating zymogen of fibrinolytic serine protease plasmin, and plasma concentration of plasminogen is estimated to be ~2 U/ml (Xue and Del Bigio, 2005). Although neurotoxicity of plasminogen in the striatum has been reported by *in vivo* experimental model (Xue and Del Bigio, 2001, 2005), we did not observe neurotoxicity of plasminogen in cortico-striatal slice cultures even at 10 U/ml, a higher concentration than that in plasma. Instead, we found that a prominent effect of plasminogen was potentiation of neurotoxicity of thrombin (30 U/ml) in the cerebral cortex. As previously demonstrated, thrombin alone can produce cortical injury at a higher concentration of 100 U/ml (Fujimoto et al., 2006). Therefore, the action of plasminogen can be viewed as lowering of the threshold of neurotoxic actions of thrombin. The plasma concentration of prothrombin, the precursor of thrombin, is estimated to be 200 U/ml (Lee et al., 1996), which means that the concentration of thrombin used in the present study (30 U/ml) is probably attainable in the case of hemorrhagic events. Plasminogen had no effect on thrombin toxicity in the striatum, which may reflect the fact that the cellular mechanisms of thrombin neurotoxicity are different between the cerebral cortex and the striatum (Fujimoto *et al.*, 2006).

We also suggest that the potentiating effect of plasminogen on thrombin neurotoxicity was mediated by plasmin generated from plasminogen processing. This view was supported by the finding that the supernatant of slice cultures treated with plasminogen exhibited plasmin activity. Moreover, cortical injury induced by a combination of thrombin and plasminogen was abolished by aprotinin, a plasmin inhibitor. Although aprotinin is a protease inhibitor with a broad spectrum, the concentration of aprotinin applied to slice cultures (20 µg/ml), corresponding to ~0.6 µM, was much lower than the concentration that inhibited thrombin (30



$\mu\text{M}$ ; Engles, 2005). Moreover, aprotinin did not abolish cortical injury and striatal shrinkage induced by a higher concentration of thrombin. Therefore, the effect of aprotinin on neurotoxicity of thrombin plus plasminogen was likely attributable to inhibition of plasmin activity.

Similar to aprotinin, tranexamic acid abolished cortical injury induced by combined application of thrombin and plasminogen, without any effect on injury induced by a high concentration of thrombin. These results indicate that the protective effect of tranexamic acid was also mediated by inhibition of plasmin(ogen) activity. There are two plausible mechanisms for the effect of tranexamic acid. Firstly, tranexamic acid may inhibit the conversion of plasminogen to plasmin. Plasminogen binds to fibrin and cell surface through lysine-binding sites located in kringle domain (Hajjar *et al.*, 1986; Wu *et al.*, 1990), which promotes the conversion of plasminogen by tissue-type plasminogen activator (Miles *et al.*, 2005; Plow *et al.*, 1995). Lysine analogs such as tranexamic acid reversibly bind to the kringle domain (Prentice, 1980), decreasing plasmin production (Ellis *et al.*, 1991). Secondly, interference of association of plasmin(ogen) with cell surface may be responsible for the effect of tranexamic acid. For example, G-protein-coupled receptors sensitive to pertussis toxin have been shown to function as a plasmin receptor in monocytes and endothelial cells (Chang *et al.*, 1993; Syrovets *et al.*, 1997). Since the responses of these cells to plasmin were inhibited by tranexamic acid, actions of plasmin may be mediated not only by its protease activity but also by its binding to unidentified cell surface receptors through kringle domain (Chang *et al.*, 1993; Syrovets *et al.*, 1997). Another report in this context has shown that  $\alpha$ -enolase is expressed on the surface of neurons as a plasminogen receptor, and that binding of plasminogen to neurons is abolished by tranexamic acid

(Nakajima *et al.*, 1994). Possible involvement of protease activity and receptors of plasmin(nogen) in potentiation of thrombin neurotoxicity remains to be addressed.

Argatroban, a thrombin inhibitor, abolished cortical injury induced by a combination of thrombin and plasminogen, confirming that protease activity of thrombin was crucial for the combined neurotoxicity. Moreover, inhibitors of ERK pathway, Src family tyrosine kinase, PKC and *de novo* protein synthesis prevented the combined neurotoxicity, whereas an inhibitor of p38 MAPK was without effect and an inhibitor of JNK exacerbated the injury. These pharmacological profiles totally mimic those of neurotoxicity of a higher concentration of thrombin (Fujimoto *et al.*, 2006). Thus, plasminogen may potentiate thrombin neurotoxicity by allowing low concentrations of thrombin to effectively recruit neurotoxic signaling mechanisms. Surprisingly, persistent ERK phosphorylation in response to plasminogen was exclusively observed in astrocytes, although thrombin-induced ERK phosphorylation has been observed mainly in neurons (Fujimoto *et al.*, 2006). These results imply that some cell-to-cell interactions between astrocytes and neurons should be involved in potentiation of thrombin neurotoxicity by plasminogen. ERK phosphorylation in astrocytes is known to trigger astrogliosis accompanied by proliferation and expression of cyclooxygenase (Brambilla *et al.*, 2002; Mandell and VandenBerg, 1999), and astrogliosis is observed in many neurodegenerative conditions (Pekny and Nilsson, 2005). Because reactive astrocytes release various inflammation-related molecules including cytokines (Hu *et al.*, 1997; Suzumura *et al.*, 2006), activation of astrocytes by plasminogen may lead to enhanced release of cytokines that make neurons vulnerable to thrombin. This possibility should also be addressed in future investigations. Finally, we cannot formally exclude the possibility that the protease activity of thrombin itself is somehow enhanced by

plasminogen/plasmin.

Potential of thrombin neurotoxicity by plasminogen in the cerebral cortex was also observed in a rat brain microinjection model, suggesting that the same cellular mechanisms of synergistic cytotoxicity of thrombin and plasminogen as those *in vitro* also operate *in vivo*. Moreover, involvement of plasminogen in hemorrhagic brain injury was ascertained by the protective effect of aprotinin against cortical injury in a collagenase-induced hemorrhage model. As demonstrated in our recent study of intrastriatal hemorrhage (Ohnishi et al., 2007), the hematoma region contained remaining viable neurons as revealed by NeuN immunoreactivity, and we observed a significant protective effect of aprotinin as the increase in the number of NeuN-positive cells. Aprotinin and tranexamic acid in the present study did not reduce the hematoma size in the cortex. Although these drugs have been clinically used to prevent blood loss (Engles, 2005; Mahdy and Webster, 2004), prior administration of high doses of aprotinin and tranexamic acid is required to prevent uncontrolled plasma extravasation *in vivo* (O'Brien *et al.*, 2000). Therefore, relatively weak neuroprotective effects of aprotinin and tranexamic acid systemically administered in the present study might be attributable to insufficient inhibition of plasmin activity by these drugs. Recently, molecules that promote blood coagulation and prevent hematoma growth, such as recombinant factor VII, attract considerable interests as a therapeutic strategy against ICH (Mayer and Rincon, 2005; Mayer *et al.*, 2005). Because inhibition of plasmin is expected to reduce hemorrhage and also to prevent neuronal injury mediated by protease activity, regulation of plasminogen/plasmin system could be an attractive therapeutic intervention.

In conclusion, we demonstrated here that plasminogen potentiated thrombin neurotoxicity in the cerebral cortex both *in vitro* and *in vivo*. These effects of plasminogen appeared to be

mediated by plasmin protease activity and ERK phosphorylation in astrocytes. Recently, exacerbation of thrombin-induced neuronal death by a combination with matrix metalloprotease-9 has been reported (Xue *et al.*, 2006). Elucidation of interactions of various protease systems with thrombin-mediated cytotoxic pathways may lead to discovery of novel therapeutic strategies for hemorrhagic brain injury

## References

- Brambilla R, Neary JT, Cattabeni F, Cottini L, D'Ippolito G, Schiller PC, Abbracchio MP (2002) Induction of COX-2 and reactive gliosis by P2Y receptors in rat cortical astrocytes is dependent on ERK1/2 but independent of calcium signalling. *J Neurochem* 83:1285-96
- Castellino FJ, Ploplis VA (2005) Structure and function of the plasminogen/plasmin system. *Thromb Haemost* 93:647-54
- Chang WC, Shi GY, Chow YH, Chang LC, Hau JS, Lin MT, Jen CJ, Wing LY, Wu HL (1993) Human plasmin induces a receptor-mediated arachidonate release coupled with G proteins in endothelial cells. *Am J Physiol* 264:C271-81
- Chen ZL, Strickland S (1997) Neuronal death in the hippocampus is promoted by plasmin-catalyzed degradation of laminin. *Cell* 91:917-25
- Donovan FM, Pike CJ, Cotman CW, Cunningham DD (1997) Thrombin induces apoptosis in cultured neurons and astrocytes via a pathway requiring tyrosine kinase and RhoA activities. *J Neurosci* 17:5316-26
- Ellis V, Behrendt N, Danø K (1991) Plasminogen activation by receptor-bound urokinase. A kinetic study with both cell-associated and isolated receptor. *J Biol Chem* 266:12752-8
- Engles L (2005) Review and application of serine protease inhibition in coronary artery bypass graft surgery. *Am J Health Syst Pharm* 62 (Suppl. 4):S9-14
- Fujimoto S, Katsuki H, Kume T, Akaike A (2006) Thrombin-induced delayed injury involves multiple and distinct signaling pathways in the cerebral cortex and the striatum in organotypic slice cultures. *Neurobiol Dis* 22:130-42
- Fujimoto S, Katsuki H, Ohnishi M, Takagi M, Kume T, Akaike A (2007) Thrombin induces striatal neurotoxicity depending on mitogen-activated protein kinase pathways in vivo.

*Neuroscience* 144:694-701

Hajjar KA, Harpel PC, Jaffe EA, Nachman RL (1986) Binding of plasminogen to cultured human endothelial cells. *J Biol Chem* 261:11656-62

Hu S, Peterson PK, Chao CC (1997) Cytokine-mediated neuronal apoptosis. *Neurochem Int* 30:427-31

Lee KR, Colon GP, Betz AL, Keep RF, Kim S, Hoff JT (1996) Edema from intracerebral hemorrhage: the role of thrombin. *J Neurosurg* 84:91-6

Mahdy AM, Webster NR (2004) Perioperative systemic haemostatic agents. *Br J Anaesth* 93:842-58

Mandell JW, VandenBerg SR (1999) ERK/MAP kinase is chronically activated in human reactive astrocytes. *Neuroreport* 10:3567-72

Mayer SA, Brun NC, Begtrup K, Broderick J, Davis S, Diringer MN, Skolnick BE, Steiner T (2005) Recombinant activated factor VII for acute intracerebral hemorrhage. *N Engl J Med* 352:777-85

Mayer SA, Rincon F (2005) Treatment of intracerebral haemorrhage. *Lancet Neurol* 4:662-72

Miles LA, Hawley SB, Baik N, Andronicos NM, Castellino FJ, Parmer RJ (2005) Plasminogen receptors: the sine qua non of cell surface plasminogen activation. *Front Biosci* 10:1754-62

Nakajima K, Hamanoue M, Takemoto N, Hattori T, Kato K, Kohsaka S (1994) Plasminogen binds specifically to  $\alpha$ -enolase on rat neuronal plasma membrane. *J Neurochem* 63:2048-57

O'Brien JG, Battistini B, Zaharia F, Plante GE, Sirois P (2000) Effects of tranexamic acid and aprotinin, two antifibrinolytic drugs, on PAF-induced plasma extravasation in

- unanesthetized rats. *Inflammation* 24:411-29
- Ohnishi M, Katsuki H, Fujimoto S, Takagi M, Kume T, Akaike A (2007) Involvement of thrombin and mitogen-activated protein kinase pathways in hemorrhagic brain injury. *Exp Neurol* 206:43-52
- Patthy L (1985) Evolution of the proteases of blood coagulation and fibrinolysis by assembly from modules. *Cell* 41:657-63
- Pekny M, Nilsson M (2005) Astrocyte activation and reactive gliosis. *Glia* 50:427-34
- Plow EF, Herren T, Redlitz A, Miles LA, Hoover-Plow JL (1995) The cell biology of the plasminogen system. *FASEB J* 9:939-45
- Prentice CR (1980) Basis of antifibrinolytic therapy. *J Clin Pathol Suppl (R Coll Pathol)* 14:35-40
- Robbins KC, Summaria L, Hsieh B, Shah RJ (1967) The peptide chains of human plasmin. Mechanism of activation of human plasminogen to plasmin. *J Biol Chem* 242:2333-42
- Schneider M, Nesheim M (2004) A study of the protection of plasmin from antiplasmin inhibition within an intact fibrin clot during the course of clot lysis. *J Biol Chem* 279:13333-9
- Suzumura A, Takeuchi H, Zhang G, Kuno R, Mizuno T (2006) Roles of glia-derived cytokines on neuronal degeneration and regeneration. *Ann NY Acad Sci* 1088:219-29
- Syrovets T, Timpler B, Rieks M, Simmet T (1997) Plasmin is a potent and specific chemoattractant for human peripheral monocytes acting via a cyclic guanosine monophosphate-dependent pathway. *Blood* 89:4574-83
- Takahashi H, Nagai N, Urano T (2005) Role of tissue plasminogen activator/plasmin cascade in delayed neuronal death after transient forebrain ischemia. *Neurosci Lett* 381:189-93

- Tsirka SE, Rogove AD, Bugge TH, Degen JL, Strickland S (1997a) An extracellular proteolytic cascade promotes neuronal degeneration in the mouse hippocampus. *J Neurosci* 17:543-52
- Tsirka SE, Bugge TH, Degen JL, Strickland S (1997b) Neuronal death in the central nervous system demonstrates a non-fibrin substrate for plasmin. *Proc Natl Acad Sci USA* 94:9779-81
- Wu HL, Chang BI, Wu DH, Chang LC, Gong CC, Lou KL, Shi GY (1990) Interaction of plasminogen and fibrin in plasminogen activation. *J Biol Chem* 265:19658-64
- Xi G, Keep RF, Hoff JT (2006) Mechanisms of brain injury after intracerebral haemorrhage. *Lancet Neurol* 5:53-63
- Xue M, Del Bigio MR (2001) Acute tissue damage after injections of thrombin and plasmin into rat striatum. *Stroke* 32:2164-9
- Xue M, Del Bigio MR (2005) Injections of blood, thrombin, and plasminogen more severely damage neonatal mouse brain than mature mouse brain. *Brain Pathol* 15:273-80
- Xue M, Hollenberg MD, Yong VW (2006) Combination of thrombin and matrix metalloproteinase-9 exacerbates neurotoxicity in cell culture and intracerebral hemorrhage in mice. *J Neurosci* 26:10281-91



## Figure legends

Fig. 1. Injury induced by thrombin and plasminogen in cortico-striatal slice cultures.

(A-D) Representative images of PI fluorescence of whole slice cultures treated with vehicle (A), 30 U/ml thrombin alone (B), 30 U/ml thrombin plus 3 U/ml plasminogen (C) and 3 U/ml plasminogen alone (D) for 72 h. Broken lines in each image indicate the striatal (left) and the cortical (right) regions, respectively. Scale bars, 1 mm. (E) A confocal image of the cortical region of a slice culture treated with thrombin (30 U/ml) and plasminogen (3 U/ml) for 72 h and immunostained for NeuN. PI fluorescence (red) was colocalized with NeuN immunoreactivity (green) as indicated by arrowheads. Scale bar, 20  $\mu$ m. (F-H) The cortical injury determined by the intensity of PI fluorescence at the center of the cortical region of slice cultures treated with increasing concentration of plasminogen (Plg) alone (F), combination of thrombin and plasminogen (G) and combination of NMDA and plasminogen (H) for 72 h. For combination with NMDA, plasminogen was applied from 24 h before and concomitantly with NMDA exposure. \*\*\*  $P < 0.001$  vs. thrombin alone. (I) The striatal shrinkage induced by thrombin and plasminogen at 72 h. PC indicates slice cultures treated with 100 U/ml thrombin as a positive control.

Fig. 2. Involvement of plasmin activity in cortical injury induced by combination of thrombin and plasminogen. (A, B) Time-dependent cleavage of S2251 reagent by serum-free medium with or without plasminogen (Plg, 3 U/ml) and plasmin (Pln, 0.1 U/ml) (A), and by supernatants of slice cultures treated with vehicle and plasminogen (Plg, 3 U/ml) (B). The amount of cleaved product of S2251 was determined by absorbance at 450 nm. (C-F) Effects of aprotinin (AP, 20  $\mu$ g/ml, C, E) and tranexamic acid (TX, 3 mM, D, F) on

cortical injury induced by a combination of 30 U/ml thrombin and 3 U/ml plasminogen (C, D) or by 100 U/ml thrombin (E, F). \*\* P < 0.01, \*\*\* P < 0.001 vs. thrombin alone, ## P < 0.01, ### P < 0.001.

Fig. 3. Effects of PD98059 (100  $\mu$ M, A), PP2 (100  $\mu$ M, B), bisindolylmaleimide (BIM, 3  $\mu$ M, C), cycloheximide (CHX, 1  $\mu$ g/ml, D), argatroban (ARG, 300  $\mu$ M, E), SB203580 (100  $\mu$ M, F), clodronate (CLO, 100  $\mu$ g/ml, G) and SP600125 (100  $\mu$ M, H) on cortical injury induced by a combination of thrombin and plasminogen. Clodronate was applied from 72 h before and during thrombin/plasminogen application. Other agents were concomitantly applied with thrombin/plasminogen. \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001 vs. thrombin alone, ## P < 0.01, ### P < 0.001.

Fig. 4. Phosphorylation of ERK induced by thrombin and plasminogen. (A) Slice cultures treated with vehicle (V), 30 U/ml thrombin alone (T), 30 U/ml thrombin plus 3 U/ml plasminogen (T+P) or 3 U/ml plasminogen alone (P) were harvested at 3 h and 24 h and homogenized. Samples were subjected to Western blot analysis with specific antibodies against phosphorylated ERK and total ERK. (B, C) Confocal microscopic images of immunofluorescence of phosphorylated ERK (green) and cell type specific markers (red) after 3 h of plasminogen (3 U/ml) exposure in the cortical region of slice cultures. Immunofluorescence of phosphorylated ERK was colocalized with GFAP (B) but not with NeuN (C). Arrowheads indicate colocalization. Scale bars, 20  $\mu$ m.

Fig. 5. Plasminogen potentiates thrombin-induced cortical neuron loss *in vivo*. (A, B)

Representative images of NeuN-immunostained coronal brain sections prepared from rats that received intracortical injection of thrombin (1 U) alone (A) and thrombin (1 U) with plasminogen (0.3 U) at 72 h (B). Scale bars, 1 mm. (C) Effect of plasminogen (0.3 U) on thrombin (1 U)-induced cortical injury. The extent of cortical injury was assessed by the injured area defined as the region with scarce NeuN immunoreactivity around the injection site. Statistical difference was evaluated by Mann-Whitney test (n=10 for each group).

Fig. 6. Involvement of plasmin in hemorrhage-induced cortical injury. (A-H)

Representative images of NeuN-immunostained coronal brain sections prepared from rats that received intracortical collagenase (0.1 U) injection followed by intraperitoneal administration of saline (2 ml/kg/day, A, B, E, F), aprotinin (2 mg/kg/day, C, G) and tranexamic acid (300 mg/kg/day, D, H) with low (A-D) and high (E-H) magnification. Panels A and E show the images of the contralateral side of the brain from a saline-administered rat as a control.

Brain sections were obtained 72 h after collagenase injection. Scale bars, 1 mm (A-D) and 50  $\mu$ m (E-H), respectively. (I) Effects of aprotinin (AP, 2 mg/kg/day) and tranexamic acid (TX, 300 mg/kg/day) on the decrease of NeuN-positive cells at the center of hematoma. \* P < 0.05 vs. saline, ### P < 0.001 vs. contralateral (Contra) cortex of saline-administered rats (n=7 – 10 for each group).













