Relative Importance of Proteinase-Activated Receptor-1 Versus Matrix Metalloproteinases in Intracerebral Hemorrhage-Mediated Neurotoxicity in Mice

Mengzhou Xue, MD, PhD; Morley D. Hollenberg, DPhil, MD; Andrew Demchuk, MD; V. Wee Yong, PhD

Background and Purpose—To reduce bleeding and damage to central nervous system tissue in intracerebral hemorrhage, the coagulant effect of thrombin is essential. However, thrombin itself can kill neurons in intracerebral hemorrhage as can the matrix metalloproteinases (MMPs), which are also elevated in this condition, in part due to thrombin-mediated activation of MMPs. It is thus important to understand and block the neurotoxic effects of thrombin without inhibiting its therapeutic outcomes. In this study, we have investigated the relative roles of proteinase activated receptor-1, a thrombin receptor, and MMPs in brain injury induced by thrombin or blood.

Methods—Mice were subjected to stereotactic intracerebral injections of saline, thrombin, and autologous blood, with or without hirudin, a thrombin inhibitor, or GM6001, an MMP inhibitor. Twenty-four hours later, tissue sections were obtained to evaluate the area of brain damage and extent of dying neurons. Data from wild-type mice were compared with results obtained with proteinase activated receptor-1 null mice.

Results—In blood-induced damage to the brain parenchyma, both hirudin and GM6001 significantly reduced injury to a comparable extent (>40%) implicating both thrombin and MMPs in neurotoxicity. In proteinase activated receptor-1 null mice, blood-induced brain damage was reduced by 22.6% relative to wild-type animals; by comparison, the blood-induced brain damage was reduced by 48.3% using GM6001.

Conclusions—The neurotoxicity of blood in intracerebral hemorrhage involves both proteinase activated receptor-1 and MMP activation, with the latter appearing more prominent in causing death. (Stroke. 2009;40:2199-2204.)

Key Words: intracerebral hemorrhage ■ MMPs ■ neurotoxicity ■ PAR1 ■ thrombin

Intracerebral hemorrhage (ICH) accounts for approximately 15% to 20% of all strokes and has a poor prognosis. Many factors contribute to brain injury after ICH, including thrombin,3,4 which comes either from resident neural cells or from prothrombin in blood. That thrombin is neurotoxic in vitro and in vivo is well recognized.3,5-7

In contrast with its deleterious effects, thrombin plays a key protective role in ICH, particularly in promoting coagulation, which is vital to prevent further bleeding.5 Recombinant activated Factor VIIa, which reduces the size of hematomas in ICH,8 acts largely by generating thrombin to facilitate clot formation.

Given the competing beneficial and detrimental effects of thrombin, it would be advantageous to define and neutralize the mechanisms by which thrombin is neurotoxic so that the important effects of thrombin in regulating clot formation are maintained. At least 2 mechanisms account for thrombin-mediated neurotoxicity. The first involves the interaction of thrombin with proteinase activated receptors (PARs)3,9,10 which are G protein-coupled receptors that regulate the cellular response to extracellular serine proteinases. Of the 4 family members, PAR1, PAR3, and PAR4 are targeted by thrombin,9,11 whereas PAR2 is activated by trypsin and other enzymes. We have found that the neurotoxicity of thrombin in neuronal cultures is partially blocked by an antagonist of PAR1.4 Others have described that PAR1 activation contributes to brain ischemic injury in mice.12,13 By activating PAR1, thrombin stimulates astrocytes and microglia.10,14

Another potential mechanism of thrombin neurotoxicity involves the matrix metalloproteinases (MMPs). Although MMPs can play positive roles in the central nervous system (CNS),15,16 the sudden elevation of several MMP members in the CNS parenchyma kill neurons.15 The accumulation of MMPs in the CNS parenchyma is implicated in producing detrimental effects in acute brain injury after ICH.17-19 Brain injury in models of ICH is reduced by treatment with inhibitors of MMPs18-20 or in mice that are deficient in MMP-3, -9, or -12.18,21,22 In the current context, the possibility that thrombin-mediated neurotoxicity may involve not only PAR activation, but also the activation of MMPs is supported...
by work demonstrating that thrombin can activate the zymogen proforms of MMPs to the active enzymes. One mechanism of thrombin activation of MMPs involves the removal of the prodomain of MMPs that folds back onto the active site to keep these enzymes in an inactive state. In this article, we sought to evaluate the relative roles of PAR1 versus MMP activity in thrombin- and ICH-mediated neurotoxicity.

Materials and Methods

Animals
All procedures were done in accordance with guidelines of the Canadian Council on Animal Care and approved by the local animal ethics committee. The adult mice of mixed gender background of matched wild-type (C57BL/6) and PAR1 null mice were used. The latter were originally developed by Johnson & Johnson Pharmaceutical Research Institute provided to us under a Material Transfer Agreement and bred at our animal care facility.

Brain Damage Induced by Autologous Blood or Thrombin
The ICH model induced by autologous blood has been detailed elsewhere. In brief, 10 μL of autologous blood obtained from the tail and collected into a syringe or 2 μL of thrombin (from human plasma, T4393; Sigma, St Louis, Mo) containing 4 U, is stereotaxically injected into the striatum. In previous work, we calculated that this concentration of thrombin could be attained after ICH injury in humans. Control mice received equal volumes of saline injected into the brain.

We considered various ways to collect blood to deliver that into the CNS parenchyma. A cardiac puncture was ruled out due to its intrusive nature, and we settled on snipping a small end piece of the tail because this is not an uncommon method to collect DNA for genotyping. This protocol was approved by the local ethics committee.

To inhibit thrombin activity in blood, groups of mice were injected intracerebrally with 10 μL blood mixed with 2 μL of hirudin (4 U, from leech, H7016; Sigma). Hirudin is a specific thrombin inhibitor that blocks all effects of thrombin, including coagulation and inflammation. Instead of hirudin, controls received 10 μL tail-derived blood mixed with 2 μL of saline intracerebrally.

To inhibit the activity of MMPs in blood and within the CNS parenchyma, groups of mice were injected intracerebrally with 10 μL of tail-derived blood mixed with 2 μL of the MMP inhibitor GM6001 (Calbiochem, La Jolla, Calif) at a final concentration of 5 μg/mL. Controls received 10 μL of blood mixed with 2 μL of vehicle (0.1% DMSO). In experiments that sought to reduce the potential neurotoxicity of intracerebrally administered thrombin, the 4 μL of 4 U thrombin was premixed with 1 μL of GM6001 (final concentration at 5 μg/mL) or vehicle (0.1% DMSO) before their administration into the CNS.

We acknowledge that the administration of GM6001 and hirudin within the blood that was used to create ICH is nonphysiological, but the results do provide proof of concept of whether a local high concentration of these agents ameliorates neurotoxicity to a comparable extent to that observed in PAR1 null mice with ICH.

Histological Examination
Twenty-four hours after intrastriatal injection of blood or thrombin, mice were euthanized and sections (6 μm) were cut. Sections from 3 levels, which included the area of the hematoma and spaced 200 μm apart, were analyzed as previously described. Hematoxylin and eosin staining was used to evaluate the area of damaged brain. Fluoro-Jade B staining was used to identify dying neurons.

Pictures of hematoxylin and eosin sections were digitalized and imagePro software was used to trace the boundary of the damaged portion of each section to tabulate the area of brain damage per section. The sum of the area of brain damage of all 3 sections per mouse was then documented. The location of brain damage was defined by the presence of blood, tissue rarefaction, or necrosis as previously described. At high magnification (40× objective), and aided by an ocular reticule, Fluoro-Jade B-positive neurons were counted in 4 fields immediately adjacent to but not including the needle site as previously described. Histological analyses were evaluated blind.

In Situ Zymography of Sections of Mouse Brain
In situ zymography is a procedure to evaluate gelatinolytic activity in situ. When the substrate for proteolytic degradation is gelatin, the technique is thought to detect the enzymatic activity of the gelatinase subfamily of MMPs, that is, MMP-2 and -9. The protocol, in brief, is as follows. A solution containing 100 μg/mL FITC-labeled DQ-gelatin (EnzChek; Molecular Probes, Eugene, Ore) in reaction buffer was applied to unfixed 14 μm cryostat tissue sections. After 3 hours at 37°C, slides were washed, mounted, and photographed for fluorescence by a Spot digital camera using a 40× objective. Where gelatinolytic activity was present in tissue, the in situ degradation of FITC-labeled DQ-gelatin resulted in emission of fluorescence at that location. We focused on areas adjacent to the hematoma within a given tissue section. Positive cells for gelatin zymography were counted in 3 photographic fields captured by the 40× objective and the sum of the 3 fields per mouse was documented.

Figure 1. Histopathologic features of ICH at 24 hours caused by the injection of autologous blood into the right striatum of mice. Hematoxylin and eosin shows that 10 μL of blood (A) and 4 μL of thrombin (B) produce extensive brain damage (arrows in A and B) in wild-type mice. The indentation on the cortical surface of the brain is due to the bone wax that is used postsurgery, and this is a feature across all mice. Fluoro-Jade B staining for dying neurons shows massive death of neurons adjacent to the hematoma in wild-type mice after blood (C) and thrombin (D) intracerebral injection. Original magnifications for A and B are 250× and C and D are 400×.
Statistical Analysis
All data are expressed as mean±SD. Intergroup comparisons were made by analysis of variance followed by Bonferroni. The differences were considered significantly when P<0.05.

Results
Brains with saline injection exhibited small collections of blood remnants and negligible edema around the needle tract in the striatum as previously described.4 After blood and thrombin injection, the irregular boundary of brain injury characterized by the presence of edema, blood debris, and necrosis was visible on hematoxylin and eosin sections and was located in the striatum (Figure 1A–B). Fluoro-Jade B staining highlighted the dying neurons (Figure 1C–D).

We used animals of both sexes because the PAR1 null mice were short in numbers and we had to resort to using all available mice. By analyzing the data post hoc of the area of brain damage at 24 hours after injection of blood into wild-type mice (11 males and 5 females from Figures 2 and 5 of the blood-induced damage plus vehicle-treated groups), we did not find differences (P>0.05, Student unpaired t test) in the magnitude of ICH injury between the sexes (2.7±0.3 mm² in males versus 3.1±0.4 in females, mean±SD of area of brain damage).

The Thrombin Antagonist, Hirudin, Significantly Reduces Brain Damage and Neuronal Death in Mouse ICH Along With Reduction of Gelatinolytic Activity
A role for thrombin in producing brain injury resulting from blood deposition is unequivocally shown by the ability of hirudin coadministered with the blood to reduce the area of brain damage and the extent of neuronal death at 24 hours (Figure 2). The percentage of the reduction of brain damage and neuronal death by hirudin was 49.0% and 43.4%, respectively, compared with the blood–saline group.

Previous work4 in this model of ICH showed an increase of pro-MMP-9 levels as well as manifestation of in situ gelatinolytic (MMP-2 and MMP-9) activity. To address whether the latter was a function of thrombin, we analyzed brain tissue around the hematoma using in situ zymography. Figure 3 shows that blood increases the number of positive cellular profiles and that the gelatinolytic activity is significantly reduced by hirudin treatment.

The Attenuation of Thrombin-Induced Brain Injury by PAR1 Deficiency and MMP Inhibitor Treatment
We addressed whether thrombin-mediated neurotoxicity in PAR1 null mice might be reduced relative to the toxicity of thrombin in wild-type mice. Figure 4 shows the extent of damaged brain areas and dying neurons 24 hours after the intracerebral injection of thrombin. In PAR1 null mice, thrombin-induced brain damage and number of dying neu-
rons was significantly lower by 26.1% and 20.6% when compared with thrombin toxicity in wild-type mice (Figure 4A–B). The data thus indicated the involvement of PAR1 in thrombin-mediated neurotoxicity. That said, the reduction in neurotoxicity seen in the PAR1 null mice was less than the reduction of blood-induced neurotoxicity caused by hirudin (40%; Figure 2). Other non-PAR1 actions of thrombin were thus hypothesized, and the impact of MMP inhibition using GM6001 was therefore studied. Figure 4C–D shows that the percentage of the reduction of damaged brain area and number of dying neurons caused by GM6001 coadministered with thrombin were 34.8% and 31.7%, respectively (Figure 4C–D). Thus, PAR1 and MMP activity both contribute to thrombin neurotoxicity. We tested this further using blood rather than thrombin as the insult.

**Brain Injury Induced by Blood Is Reduced Less Profoundly by PAR1 Deficiency Than by an MMP Inhibitor**

After the injection of 10 μL of blood into the CNS, the blood-induced brain damage area and the number of Fluoro-Jade B-positive cells in the PAR1 null mice were lower by 22.6% and 17.2% compared with the damage in the wild-type mice (Figure 5A–B). A greater reduction in neurotoxicity resulted from the administration of the MMP inhibitor, GM6001 (a reduction of the damage area and number of Fluoro-Jade B-positive cells of 48.3% and 42.6%, respectively; Figure 5C–D).

**Discussion**

The main findings of our study were that for blood-induced neurotoxicity in mice (1) inhibition of MMP activity was as effective as blocking the action of thrombin by hirudin; and (2) that although thrombin contributed to neurotoxicity in the model through PAR1 activation, its ability to activate MMPs may have a greater impact on neurotoxicity.

ICH causes brain injury through many mechanisms, including edema, secondary ischemic damage, and elevation of thrombin and MMPs. Other factors present in blood such as heme released from red blood cell lysis28 add to brain damage. Thus, blood should produce injury that would be more extensive than that caused by a single thrombin injection. This is evident from the quantitation of the size of brain damage produced by blood (3 mm²; Figure 2) versus thrombin (2 mm²; Figure 4) in wild-type mice.

Many MMP members are elevated in human29,30 and rodent ICH18,21 and MMP-3 and -9 kill neurons.4,22 MMP-3, -9, and -12 null mice have lesser degrees of damage resulting from ICH,4,21,22 although the opposite result for MMP-9 has also been reported.31,32 These data indicate that inhibition of MMPs represents a practical therapeutic strategy for patients with ICH, although therapy would need to be restricted to a short timeframe not to interfere with the delayed beneficial remodeling and repair mechanisms of MMPs.15,16

The presence of thrombin within the CNS is of particular importance in ICH because its ability to convert fibrinogen to fibrin, trigger platelet aggregation, and cause vasoconstriction...
would all be of benefit in preventing further hemorrhage. These hemostatic actions of thrombin are, however, offset by the ability of thrombin to be neurotoxic.5–7,27 The untoward effects of thrombin in the setting of ICH brain injury can be attenuated by the administration of the thrombin antagonist hirudin,7,27 but the complete neutralization of thrombin action attenuated by the administration of the thrombin antagonist hirudin,7,27 but the complete neutralization of thrombin action would also eliminate its beneficial actions.

Because thrombin is a double-edged sword, it is important to understand the mechanisms of its neurotoxicity and our results implicate PAR1. Targeting PAR1 by antagonists or inhibiting its downstream effects such as signaling through phosphoinositol-3-kinase53 or potentiation of N-methyl D-aspartate receptor function54 could represent avenues for future therapies to ameliorate the toxic consequences of thrombin in ICH. Notwithstanding, our data suggest that the untoward effects of thrombin in the ICH model involve additionally the MMPs and their activity would have to be neutralized.

In evaluating the impact of GM6001, this MMP inhibitor reduced neurotoxicity of blood more effectively than that of thrombin (compare Figures 4 and 5). This differential suggests that there are other MMP-elevating agents in blood that can mediate toxicity. Among these are several inflammatory cytokines and chemokines in blood that are promoters of MMP expression by various cell types.55

In summary, our results demonstrate that although PAR1 and MMP activation both contribute to the neurotoxicity of blood, inhibition of MMP activity has a greater impact on reducing neurotoxicity than does the absence of PAR1 in the ICH model. Thus, the targeted and short-term use of MMP inhibitors alone may possibly represent a therapeutic modality for the treatment of hemorrhagic stroke in humans. The MMP inhibitors could potentially augment the efficacy of thrombin-generating agents to promote hemostasis while minimizing thrombin-induced neurotoxicity.

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Disclosures
None.

References


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