Effects of Thrombin Inhibitor on Thrombin-Related Signal Transduction and Cerebral Vasospasm in the Rabbit Subarachnoid Hemorrhage Model

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Background and Purpose—Thrombin is activated in the cerebrospinal fluid (CSF) after a subarachnoid hemorrhage (SAH). However, the relationship between thrombin and cerebral vasospasm has not yet been fully established. The aim of this study was to investigate the possibility of thrombin as a causative factor for cerebral vasospasm and to delineate the signal transduction mechanism that results in thrombin-inducing sustained vasoconstriction in cerebral vasospasm.

Methods—In the SAH group, SAH was simulated by the 2-hemorrhage rabbit model. In the treatment group, antithrombin III (AT-III) was injected into the cisterna magna just before production of the SAH. CSF samples were obtained serially to measure D-dimer with latex photometric immunoassay. On day 4, the basilar artery was excised after perfusion-fixation. The degree of cerebral vasospasm was evaluated by measuring the cross-sectional area of each basilar arterial lumen, and the expression of mitogen-activated protein kinase (MAPK) in the vascular wall was examined with an immunohistochemical technique.

Results—In the treatment group, the value of D-dimer on day 4 was 0.83 ± 0.07 μg/mL, which was statistically significantly lower than that in the nontreated SAH group (2.49 ± 0.09 μg/mL, P < 0.01). The cross-sectional area of the arterial lumen in the treatment group was 3.67 × 10^5 ± 1.58 × 10^4 square pixels, which was statistically significantly larger than that in the nontreated SAH group (2.60 × 10^5 ± 2.29 × 10^4 square pixels; P < 0.01). MAPK was detected diffusely in the vascular smooth muscle cell layer in the nontreated SAH group, but it was absent in the treatment group.

Conclusions—Inhibition of thrombin activity leads to amelioration of cerebral vasospasm and suppression of MAPK diphosphorylation. This suggests that thrombin and its related signal transduction, including the MAPK cascade, appear to play an important role in the pathogenesis of cerebral vasospasm after SAH. (Stroke. 2003;34:1497-1500.)

Key Words: subarachnoid hemorrhage ■ thrombin ■ vasospasm ■ rabbits

Cerebral vasospasm after an aneurysmal subarachnoid hemorrhage (SAH) is characterized by the prolonged and reversible contraction of the cerebral arteries.1,2 This vasospasm is 1 of the most important factors affecting the functional prognosis of patients. Although various spasmodens and origins have been studied, the pathogenesis of vasospasm remains unclear.3

Recently, it has been suggested that thrombin may play an important role in the pathogenesis of vasospasm because several clinical studies have revealed that thrombin activity in the cerebrospinal fluid (CSF) after SAH is significantly elevated in patients with symptomatic vasospasm.4 However, details of how thrombin is associated with the pathogenesis of vasospasm have not been fully established, although a single study has revealed that argatroban, a synthetic and specific thrombin inhibitor, could prevent cerebral vasospasm in a rabbit SAH model.5 Although several in vitro studies have shown that thrombin induces a sustained contraction through some intracellular signaling pathway,6 the detailed mechanisms of this thrombin-induced sustained contraction have not been clarified in vivo, especially as it relates to cerebral vasospasm.

The aims of this study were to investigate, through the use of the double-hemorrhage rabbit model,7,8 the possibility that thrombin plays a role in vasospasm as a spasmoden and to delineate the mechanism of the signal transduction that causes the sustained vasoconstriction in cerebral vasospasm. Antithrombin III (AT-III) was injected into the cisterna magna to inhibit thrombin activity. The prevention of cerebral vasospasm by AT-III and associated changes in the regulation of mitogen-activated protein kinase (MAPK) as a marker of the signaling pathway were examined.

Materials and Methods
All experimental protocols were approved by the Hirosaki University Animal Research Committee. Twenty Japanese white rabbits weighing 2.8 to 3.3 kg were used. All animals were randomly assigned to 4 groups: group 1 (SAH), SAH was produced and animals were given an intracisternal injection of vehicle (distilled water); group 2...
(SAH-AT-III), SAH was produced and the animals were given an intracisternal injection of AT-III; group 3 (untreated controls), animals served as an untreated normal control; and group 4 (treated control), animals were subjected to an intracisternal injection of AT-III without the production of SAH to serve as a treated control.

Production of SAH
In groups 1 and 2, SAH was produced according to the double-hemorrhage method. With a 27-gauge butterfly needle, pentobarbital (30 mg/kg) was injected into the central ear vein over 1 to 2 minutes. After anesthesia, under spontaneous breathing, a 23-gauge butterfly needle was percutaneously placed in the cisterna magna, and CSF (1.0 to 1.8 mL) was aspirated under aseptic technique before each blood injection. The central ear artery was cannulated to obtain autologous arterial blood. Autologous nonheparinized arterial blood was injected into the cisterna magna over 1 to 2 minutes. Then, the animals were placed in a 30° head-down tilted position for 15 minutes to ensure that the blood spread into the basal cistern. Forty-eight hours after the first SAH, a second one was produced in the same manner as the first.8

Injection of AT-III or Vehicle
In group 2, AT-III was administered before each blood injection to inhibit thrombin activity in the CSF. We dissolved 25 U AT-III in distilled water (total volume, 0.5 mL) and injected it into the cisterna magna over 1 to 2 minutes. In group 1, instead of injecting AT-III, we injected 0.5 mL distilled water into the cisterna magna in the same manner as in group 1 before each blood injection.

In group 4, an intracisternal injection of AT-III was given twice (same procedure as used in group 2) but without the production of SAH so that this group could serve as a treated control.

Biochemical Evaluation
For the purpose of evaluating thrombin activity, we measured the levels of d-dimer in the CSF 3 times in groups 1, 2, and 4. CSF samples were obtained from the cisterna magna just before the injection of AT-III or vehicle on days 0 and 2. A CSF sample was also obtained from the cisterna magna just before the animals were killed on day 4. In group 3, CSF was taken once, and the animal was killed. All samples were immediately stored at −80°C until analysis.10 d-Dimer was assayed with latex photometric immunoassay.

Histological Evaluation
In groups 1, 2, and 4, perfusion-fixation was performed on day 4 after the CSF was sampled for the evaluation of d-dimer. Animals were then anesthetized deeply with 100 mg/kg pentobarbital, the thorax was opened, and a cannula was immediately inserted into the ascending aorta via the left ventricle. Perfusion was performed at 75 mm Hg with 300 mL heparinized physiological saline (5000 U/500 mL), followed by 500 mL of phosphate-buffered 4% paraformaldehyde (pH 7.4). Finally, the brain was carefully removed so as not to stretch and injure the basilar artery. Group 3 underwent maldehyde (pH 7.4). Finally, the brain was carefully removed so as not to stretch and injure the basilar artery. Group 3 underwent maldehyde (pH 7.4). Finally, the brain was carefully removed so as not to stretch and injure the basilar artery.

The entire length of the basilar artery was divided into 3 sections. We examined the middle section, stretching from a point 2 mm from the distal end of the basilar artery to 3 mm from the proximal end. The tissue was dehydrated in graded alcohol and embedded in paraffin. All 6-μm-thick sections were cut vertically, mounted on a glass slide, and stained with hematoxylin-eosin (HE) or with immunohistochemical stain.

Cerebral vasospasm was evaluated using the HE-stained sections. The cross-sectional area of each basilar artery lumen was measured with the National Institutes of Health image program (version 1.61). Data are expressed as square pixel (SP). Morphometric investigations of the arterial cross sections from the above portion of the basilar artery were performed.

Immunohistochemical staining was performed to evaluate MAPK activity with anti-MAPK (diphosphorylated ERK1&2, Labeled

Figure 1. d-Dimer levels in all groups at 3 sampling times (days 0, 2, and 4). Values are presented as mean±SE. d-Dimer was not detected in the CSF taken on day 0 in groups 1, 2, and 4 and at the time just before death in group 3. On day 4, there was a significant difference between groups 1 and 2 (P<0.01).

Streptavidin Biotin kit, Dako). The drug was diluted to 1:100 with 1% bovine serum albumin/phosphate-buffered solution (BSA/PBS) (BSA , 1 g, pH 7.4, 0.01 mol/L, 100 mL). A total of 100 μL anti-MAPK was used for each section and was incubated for 1 hour at room temperature. The sections were later washed with PBS and incubated with a 1:100 dilution of biotin-labeled anti-mouse immunoglobulin goat antibody for 1 hour at room temperature. Then, the sections were washed with PBS and incubated with peroxidase-labeled streptavidin for 1 hour at room temperature. DAB-H2O2 solution was used to visualize MAPK. MAPK expression in the vascular smooth muscle layer was reviewed by 3 blinded independent observers. The intensity in the vascular smooth muscle layer was graded on a 3-point scale, from 0 (least intense) to 2 (most intense).

Statistical Analysis
The Mann-Whitney U test was used to compare the intensity score among the groups. To compare 2 unpaired groups, the t test was used. Multiple comparisons of the data were analyzed by analysis of variance (ANOVA, Bonferroni’s method).

Results
Thrombin Activity (d-Dimer)
The cutoff value of d-dimer in the CSF is 0.5 μg/mL, and values >0.5 μg/mL can be detected. d-Dimer was not detected in the CSF taken on day 0 in groups 1, 2, and 4 and before perfusion-fixation in group 3. In group 4, d-dimer levels were <0.5 μg/mL on days 2 and 4. In group 1, d-dimer levels were 2.23±0.05 and 2.49±0.09 μg/mL on days 2 and 4, respectively. In contrast, in group 2, d-dimer levels were 2.01±0.14 and 0.83±0.07 μg/mL on day 2 and 4 (Figure 1). On day 2, there was no statistically significant difference between groups 1 and 2 in thrombin activity. However, a statistically significant difference (P<0.01) in thrombin activity between groups 1 and 2 was seen on day 4.

Evaluation of Cerebral Vasospasm
The mean basilar artery cross-sectional area was 4.56×103±0.76×103 SP in group 3 and 4.61×103±0.47×103 SP in group 4 (P=NS). Thus, the mean basilar artery cross-sectional area was not affected by the injection of AT-III without SAH production. The average cross-sectional

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area was 2.60 $\times 10^{3}$–2.29 $\times 10^{4}$ SP for group 1 and 3.67 $\times 10^{3}$–1.58 $\times 10^{4}$ SP for group 2 (Figure 2). The change in lumen cross-sectional area was $\approx 25\%$ (with a 12% reduction in vessel diameter) for groups 1 and 2 compared with group 3. In addition, there was a statistically significant difference in the mean cross-sectional area between groups 1 and 2 ($P < 0.01$). No significant difference was found between groups 3 and 4.

MAPK Activity

In both groups 3 and 4, MAPK was not detected in the smooth muscle cells, which was evaluated as a score of 0. In group 1, MAPK was detected diffusely at the vascular smooth muscle layer (Figure 3C). Immunoreactivity was better visualized compared with the background, and the average score was 1.8. In contrast, all sections of group 2 showed lower MAPK immunoreactivity (Figure 3D), with an average score of 0.2. With the Mann-Whitney $U$ test, a statistically significant difference in MAPK scores was found between groups 1 and 2 ($P < 0.01$; the Table).

Discussion

Although it is known that prothrombin exists in the CSF, thrombin has not yet been confirmed to be present in normal CSF. Nevertheless, once bleeding into the subarachnoid space occurs, thrombin is activated rapidly and remains at a high level because a firm, persistent fibrin network is produced through activation of the coagulation system in the subarachnoid space. Furthermore, CSF thrombin is only minimally inactivated by the AT-III found in circulating blood and by the thrombomodulin found in vascular endothelial cells. A previous study has shown that post-SAH CSF thrombin activity is correlated with the persistence of blood and development of vasospasm.

After SAH, it has been found that levels of thrombin–AT-III complex and prothrombin fragment F1 + 2, both molecular markers of CSF thrombin activation, are elevated and that these levels correlate well with both the clinical severity at the onset of SAH and the occurrence of cerebral vasospasm. In the present study, CSF thrombin activity was evaluated by measuring $D$-dimer. $D$-Dimer, the decomposition product of fibrin, reflects thrombin activity, as do the thrombin–AT-III complex, prothrombin fragment F1 + 2, and fibrinopeptide A. After SAH, $D$-dimer is found in the CSF with a high sensitivity. Results of this study revealed that increased CSF thrombin activity was seen in the SAH plus distilled water (untreated control) group.

To inhibit thrombin activity, we used AT-III. AT-III, with a molecular weight of $\approx 59,000$ U, is an endogenous plasma glycoprotein that inhibits a number of serine proteases.
Results of the present study showed that intrathecal administration of AT-III significantly decreased the CSF levels of D-dimer compared with the SAH control group. The decrease in D-dimer resulting from intrathecal administration of AT-III paralleled the increase in cross-sectional areas, which demonstrated the improvement of the cerebral vasospasm. Therefore, thrombin in the CSF may be involved in the pathogenesis of cerebral vasospasm.

Smooth muscle contraction was thought to be caused by myosin light-chain phosphorylation induced by an increase in intracellular Ca2⁺ concentration, followed by the sliding of myosin and actin proteins. However, several studies have revealed that myosin light-chain phosphorylation is not up-regulated and that high-energy phosphates decline during cerebral vasospasm. Thus, mechanisms that can cause vasospasm by processes other than that associated with myosin light-chain phosphorylation must be considered. Therefore, various intracellular signal transduction pathways have been investigated in association with cerebral vasospasm.3,6

Recently, the MAPK (ERK1&2) cascade has been thought to play an important role in cerebral vasospasm. MAPK is a family of serine/threonine protein kinases involved in cell growth, transformation, and proliferation through the activation of transcription factors and target genes. It also plays an important role in prolonged smooth muscle contraction by phosphorylating caldesmon, which is a thin filament-associated protein that inhibits α-actin. Zubkov et al19 revealed that tyrosine kinase and MAPK cascades play a role in hemolysate-induced contraction of rabbit cerebral arteries, a process that is independent of intracellular Ca2⁺ concentration. The MAPK cascade is thought to be upregulated by tyrosine kinase activated as a result of growth factors such as platelet-derived growth factor18 and by protein kinase C activation as a result of a G protein–coupled receptor agonist.3

An in vitro study using bovine aorta showed that thrombin caused sustained contraction through the activation of the MAPK cascade.6 However, the role of MAPK cascade activation in thrombin-induced cerebral vasospasm has not been evaluated in vivo.

Previous studies have revealed that in situ distribution of the active, dual phosphorylated form of MAPK (ERK1&2) could be visualized in a wide range of organisms by immunohistochemistry with a specific monoclonal antibody.19 In the present study, a similar technique was used to evaluate MAPK activity in the arterial wall. Results showed that MAPK detection was markedly increased in the vascular smooth muscle cell layer after SAH production but that it was diminished through the suppression of thrombin activity achieved by the intrathecal administration of AT-III. Therefore, sustained contraction during cerebral vasospasm may be attributed partially to activation of the MAPK cascade induced by thrombin. We propose that triggering of the MAPK cascade results in the phosphorylation of caldesmon, which plays an important role in prolonged smooth muscle contraction. In short, thrombin stimulates tyrosine phosphorylation of growth factor receptors, and the activation of the receptor tyrosine kinases then stimulates the MAPK cascade, which phosphorylates caldesmon and leads to contraction.

Clearly, the detailed mechanism of cerebral vasospasm in association with signal transduction, including the MAPK cascade, should be investigated in the future. Yet, the results of this study suggest that the inhibition of thrombin activity could be a powerful preventive therapy for cerebral vasospasm.

References
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