Delayed Argatroban Treatment Reduces Edema in a Rat Model of Intracerebral Hemorrhage

Takuji Kitaoka, MD; Ya Hua, MD; Guohua Xi, MD; Julian T. Hoff, MD; Richard F. Keep, PhD

Background and Purpose—Studies indicate that thrombin plays an important role in intracerebral hemorrhage (ICH)-induced edema formation. Although thrombin is produced as the blood clots, it may be bound to fibrin and only gradually released from the clot. The time window for administration of a thrombin inhibitor to reduce ICH-induced edema is unknown. Whether this time window extends beyond the period when a thrombin inhibitor might exacerbate rebleeding is also unknown.

Methods—This study examines (1) whether argatroban, an inhibitor of both free and fibrin-bound thrombin, can reduce edema formation after intracerebral infusion of 100 μL of blood in the rat; (2) the therapeutic time window for argatroban; and (3) whether argatroban promotes rebleeding in a model in which ICH was induced by intracerebral injection of collagenase.

Results—Intracerebral infusion of blood caused a marked increase in perihematomal water content. Intracerebral injection of argatroban 3 hours after ICH caused a significant reduction in edema measured at 48 hours (80.9±1.0% versus 82.6±0.8%; P < 0.01). The systemic administration of high-dose argatroban (0.9 mg/h) starting 6 hours after ICH also significantly reduced edema (80.3±1.1% versus 82.0±1.3% in vehicle controls; P < 0.05). There was no protection when the onset of argatroban administration was delayed to 24 hours after ICH or if a lower dose of argatroban (0.3 mg/h) was used. Argatroban did not increase collagenase-induced hematoma volume when given into the clot after 3 hours or given systemically at 6 hours.

Conclusions—Our data suggest that argatroban may be an effective therapy for ICH-induced edema. (Stroke. 2002;33:3012-3018.)

Key Words: antithrombins ■ brain edema ■ intracerebral hemorrhage ■ thrombin ■ rats

Intracerebral hemorrhage (ICH) represents 8% to 15% of all strokes in Western populations and 20% to 30% in Oriental populations. The 30-day mortality rate is 35% to 50%, and most survivors are typically neurologically disabled, with only 20% of patients becoming independent at 6 months.

There is currently no definitive treatment that improves ICH outcome. Neurological deficits arise not only from direct tissue destruction but also from widespread effects due to cerebral edema. Moreover, it has been suggested that perilesional edema is poorly responsive to surgical clot evacuation.

Previous studies have demonstrated that toxic components, including thrombin, released from a developing blood clot may account for perihematomal edema formation. Direct intracerebral infusion of thrombin causes inflammation, edema, reactive gliosis, apoptosis, and scar formation. Thrombin is generated as blood clots, and evidence indicates that thrombin plays an important role in ICH-induced edema formation. In those experiments, thrombin inhibitors hirudin or α-N-(2-naphthalenesulfonyl)-glycyl-(D)-4-aminophenylalanyl-piperidine (NAPAP), administered 5 minutes after placement of an intracerebral hematoma in a rat, greatly reduced ICH-induced edema formation. Evidence from humans on anticoagulants also suggests that the clotting cascade is involved in ICH-induced edema formation.

Although thrombin formation occurs rapidly after ICH, enzymatically active thrombin may remain within a clot associated with fibrin, leading to a delayed release of thrombin into surrounding brain tissue. Thus, a long therapeutic time window for a thrombin inhibitor may exist. This is important because administration of a thrombin inhibitor too soon after an ICH may lead to rebleeding with potentially devastating effects. Thrombin inhibitors vary in their ability to inhibit clot-bound thrombin. Thus, Berry et al found that hirudin and heparin were >1000-fold less effective in inhibiting clot-bound compared with soluble thrombin. In contrast, the potency of another inhibitor, argatroban, was reduced only 2-fold. Argatroban is currently approved in Japan for treatment of peripheral artery occlusive disease and acute ischemic stroke and in the United States for anticoagulant therapy in heparin-induced thrombocytopenia.

The present study had 2 goals: (1) to determine whether delayed administration of argatroban (intracerebral or systemic)
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Argatroban could reduce edema formation in a rat model of ICH involving injection of autologous blood into the caudate nucleus and (2) to examine whether argatroban administration would increase rebleeding in a collagenase model of ICH.

Materials and Methods

Animal Preparation and Intracerebral Infusions

The animal protocols were approved by the University of Michigan Committee on the Use and Care of Animals. A total of 93 male Sprague-Dawley rats (Charles River Laboratories), each weighing 300 to 400 g, were used for all experiments. Rats were allowed free access to water and food. The animals were anesthetized with pentobarbital (50 mg/kg IP), and the right femoral artery was catheterized to monitor arterial blood pressure and to sample blood for intracerebral infusion. Blood pH, PaO2, PaCO2, hematocrit, and glucose levels were monitored. Body temperature was maintained at 37°C with the use of a feedback-controlled heating pad.

Rats were then positioned in a stereotaxic frame (Kopf Instruments). A cranial burr hole (1 mm) was drilled near the right coronal suture 3.5 mm lateral to the midline. A 26-gauge needle was inserted stereotaxically into the right basal ganglia (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 3.5 mm lateral to the bregma). This needle was used for infusions of autologous blood or collagenase, the 2 ICH models used in this study.

Experimental Groups

This study was performed in 3 parts. Part 1 evaluated the effect of direct administration of argatroban (provided by Mitsubishi Pharma Corporation) to an intracerebral hematoma on brain edema formation. Two groups of animals were studied. In each, rats (n=6) received a 100-μL infusion of autologous blood into the right basal ganglia at a rate of 10 μL/min with the use of a microinfusion pump (Harvard Apparatus). After infusion, the needle was removed, and skin incisions were closed. Three hours later, the animals were reanesthetized, and a Hamilton syringe was inserted with the use of the same stereotaxic coordinates. Twenty microliters of saline±argatroban (0.5 μg/mL) was then infused over 10 minutes. The needle was then removed, the skin was closed, and the animals were allowed to recover. Animals were killed 48 hours after blood infusion for measurement of brain water and ion contents.

Part 2 investigated the effect of systemic argatroban administration on ICH-induced edema formation. For these experiments, argatroban was dissolved in physiological saline solution containing hydrochloric acid (pH 1.2 to 1.8). Physiological saline adjusted to the same pH with hydrochloric acid was used as the vehicle. These solutions were used to fill osmotic minipumps (ALZET model 2 ML1, Alza Corporation) that deliver 10 μL/h; the minipumps were then implanted intraperitoneally. Five sets of experiments were performed. All animals were killed 48 hours after ICH or saline infusion for edema and ion measurements. In the first set of experiments, animals received an intracerebral infusion of 100 μL autologous blood. After 6 hours they were reanesthetized and implanted with the osmotic minipumps delivering vehicle (n=6) or argatroban (0.9 mg/h per rat; n=5). The animals were then allowed to recover. In the second set of experiments, osmotic minipumps were not implanted until 24 hours after the ICH. There were 5 and 6 animals in the vehicle and argatroban groups, respectively. In the third set of experiments, a lower dose of argatroban was used (0.3 mg/h per rat), and the infusion was started at 6 hours after ICH. There were 6 animals in the vehicle and argatroban groups. In the fourth set of experiments, naïve rats were implanted with osmotic minipumps delivering vehicle (n=5) or argatroban (0.9 mg/h per rat; n=5). In the fifth set of experiments, animals received an intracerebral infusion of 100 μL saline. After 6 hours they were reanesthetized and implanted with the osmotic minipumps delivering vehicle (n=7) or argatroban (0.9 mg/h per rat; n=6).

Part 3 examined whether argatroban would induce rebleeding in a collagenase model of ICH. Rats received a 2-μL injection (at 0.2 μL/min) of saline containing 0.4 U bacteria collagenase (type VII, Sigma Chemical Co). Once the infusion was completed, the Hamilton syringe was left in place for 5 minutes. These animals were used for 2 sets of experiments. In the first set, the animals were reanesthetized after 3 hours, when they received an intracerebral infusion of 20 μL saline or 20 μL saline+argatroban (0.5 μg/mL) over 10 minutes (n=6 per group). The stereotaxic coordinates were the same as used for the collagenase injection. In the second set of experiments, the animals were reanesthetized after 6 hours and implanted with an osmotic minipump intraperitoneally delivering either saline (10 μL/h) or saline+argatroban (0.9 mg/h per rat). There were 6 rats per group. For both sets of experiments, the animals were decapitated 48 hours after the initial collagenase injection for determination of hematoma volume and brain hemorrhage area.

Analytical Methods

Brain Water and Ion Contents

Forty-eight hours after ICH, animals were reanesthetized and decapitated. The brains were removed, and a coronal brain slice (3 mm in thickness) 4 mm from the frontal pole was taken. The brain slice was divided into the 2 hemispheres, and each hemisphere was dissected into cortex and basal ganglia (a total of 4 samples). The ipsilateral basal ganglia sample surrounds the hematoma and is referred to as perihematomat. The cerebellum also served as a control. Samples were immediately weighed on an electronic analytical balance (model AE100, Mettler Instrument Co) to obtain the wet weight. Brain samples were then dried at 100°C for 24 hours to obtain the dry weight, and the water content was determined as (Wet Weight−Dry Weight)/Wet Weight. The dehydrated samples were digested in 1 mL of 1 mol/L nitric acid for 1 week before sodium and potassium content determination by flame photometer (model IL 943, Instrumentation Laboratory Inc.). Chloride was measured with the use of a digital chloride meter (model 4420 to 5000, Haake Buchler Inc.). Ion content was expressed in milliequivalents per kilogram of dehydrated brain tissue (mEq/kg dry wt).

Morphometric Measurement of Hemorrhage Volume

Animals were decapitated, and the brains were rapidly removed and sectioned coronally at 2-mm intervals. With the use of a computerized image analysis system (NIH Image, version 1.61), the hemorrhage area for each section was measured. The total hematoma volume was calculated by summing the clot area in each section and multiplying by the distance between sections.

Spectrophotometric Hemoglobin Assay

The ipsilateral cerebral hemisphere was collected from each animal. Distilled water (1 mL) was added to each hemisphere, followed by homogenization for 30 seconds, sonication on ice with an ultrasonicator for 1 minute, and centrifugation at 13 000 rpm for 30 minutes. The hemoglobin containing supernatant was collected, and 80 μL of Drabkin’s solution was added to a 20-μL aliquot. Fifteen minutes later the optical density of the solution at 550-nm wavelength was measured to assess hemoglobin content.

Argatroban Concentrations

Plasma argatroban concentrations were determined by high-performance liquid chromatography by a method described previously.

Statistical Analysis

All data in this study are presented as mean±SD. Data were analyzed by unpaired Student’s t test. A 2-tailed probability value of <0.05 was taken to indicate a significant difference.

Results

Physiological Parameters

There were no significant differences in blood gases, blood pH, blood glucose, hematocrit, and blood pressure among the experimental groups measured at the time of ICH induction. Plasma argatroban concentrations at 48 hours were...
0.25 ± 0.10 and 0.59 ± 0.30 μmol/L in rats receiving argatroban infusions at 0.3 and 0.9 mg/h per rat (n = 12 and n = 20), respectively.

Brain Water Content
Intracerebral injection of argatroban 3 hours after ICH caused a significant reduction in perihematomal edema formation measured 48 hours after ICH. Thus, in the ipsilateral basal ganglia, water content was 80.9 ± 1.0% in the argatroban group compared with 82.6 ± 0.8% in the vehicle group (Figure 1). Perihematomal edema is associated with an increase in the sum of the major brain inorganic ions. The reduction of brain edema formation with argatroban was associated with reduced total ion accumulation in the treatment group (Figure 1). Argatroban did not significantly alter the water content of tissues distant from the site of blood injection.

Intraperitoneal administration of the high dose (0.9 mg/h) of argatroban starting 6 hours after ICH caused a significant reduction in perihematomal water content measured 48 hours after ICH. Thus, in the ipsilateral basal ganglia, brain water content was 80.3 ± 1.1% and 82.0 ± 1.3% in the argatroban and vehicle-treated rats, respectively. Argatroban treatment also had a small but significant effect on brain water content in the contralateral basal ganglia (Figure 2). There is some tracking of edema from ipsilateral to contralateral hemispheres in this rat model.14 Brain edema reduction in ipsilateral basal ganglia was associated with reduced total ion accumulation (Figure 2). There was no effect of argatroban treatment on brain water content in tissues, such as the cerebellum, distant to the site of blood injection (Figure 2). Intraperitoneal infusion of argatroban from 6 hours after ICH at a lower dose (0.3 mg/h) failed to reduce perihematomal edema formation (Table). Similarly, delaying the onset of argatroban (0.9 mg/h) infusion from 6 to 24 hours after ICH meant that argatroban ceased to be effective in reducing perihematomal edema formation (Table).

Intraperitoneal infusion of argatroban at the high dose (0.9 mg/h) per rat) had no effect on brain water content in rats in which no needle had been placed within the brain (Table). However, argatroban had a slight but significant effect on brain water content in animals that had a needle placement and an injection of saline.

Safety
Unlike in a human ICH, the rat ICH model used in the previous studies does not have a ruptured blood vessel as the source of the hematoma. Thus, although argatroban might reduce the adverse effects of parenchymal blood, such a benefit might be outweighed if it induced rebleeding. To examine whether argatroban might cause rebleed-
ing, the collagenase ICH model of Rosenberg et al. was used.

Hematoma volume was assessed morphometrically 48 hours after ICH in rats receiving either argatroban or vehicle. Argatroban did not increase collagenase-induced hematoma volume when given into the clot at 3 hours (Figure 3) or given systemically at 6 hours at a rate of 0.9 mg/h (Figure 4) compared with vehicle-treated rats. Examples of the hematomas are also shown in Figures 3 and 4. There was no mortality in any of these experiments.

Brain hemoglobin content was also used to assess hematoma mass. Again, there were no differences in hemoglobin

<table>
<thead>
<tr>
<th>Group</th>
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<th>Delay of Treatment</th>
<th>Dose, mg/h</th>
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<td>Ipsilateral Basal Ganglia</td>
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Values are mean±SD; n=5 to 7. Basal ganglia water contents were determined 48 hours after ICH, sham operation, or drug treatment in naive rats.

*Difference between argatroban- and vehicle-treated rats at P<0.01 level.

Figure 3. Hematoma volume (A) and brain hemoglobin (optical density) (B) 48 hours after intracerebral infusion of collagenase. Three hours after ICH, rats received an intracerebral injection of argatroban (20 μL of 0.5 μg/mL) or saline. Values are mean±SD. An example of brain slices in each group is shown.
content between the rats that received argatroban into the clot at 3 hours or rats that were given argatroban systemically at 6 hours (0.9 mg/h) and vehicle-treated controls (Figures 3 and 4). Since the hemoglobin concentration in the blood was the same in the argatroban and vehicle groups, this indicates that the clot mass was not significantly different.

Discussion

Multiple mechanisms are involved in inducing perihematomal edema formation, but thrombin appears to be an important mediator.\(^7,8,16\) The present study indicates that administration of argatroban, even when delayed by 6 hours, can reduce ICH-induced edema formation. Argatroban administered systemically after 6 hours did not induce rebleeding in collagenase-induced ICH, suggesting that argatroban could be a potential therapeutic agent for ICH.

Therapeutic Time Window

In previous rat studies we have shown that injections of 2 thrombin inhibitors, hirudin and α-NAPAP, into an intracerebral hematoma within 5 minutes of blood injection can significantly reduce perihematomal edema formation.\(^7,8\) In contrast to normal blood, injection of anticoagulated blood (with heparin) does not cause perihematomal edema in rat or pig,\(^9\) and evidence indicates that anticoagulated blood is not edematogenic in humans as well.\(^9\) Thrombin can induce cytotoxic effects on brain parenchymal cells,\(^17,18\) blood-brain barrier disruption,\(^18\) and an inflammatory response.\(^19\) These effects may contribute to the edematogenic properties of thrombin. Thrombin-induced edema does not appear to be related to ischemia because intracerebral thrombin injections do not reduce cerebral blood flow.\(^18\)

These previous studies have demonstrated that prior thrombin inhibition or thrombin inhibition immediately after ICH can reduce ICH-induced edema formation. The present study demonstrates that delayed intracerebral or systemic administration of the thrombin inhibitor argatroban can also reduce perihematomal edema formation. ICH-induced brain edema formation appears to result from an accumulation of sodium and chloride within the brain that outweighs a loss in tissue potassium.\(^14\) These ion shifts are also reduced by delayed treatment with argatroban.

Although thrombin is formed almost immediately after an ICH, there are potential reasons why delayed administration...
of a thrombin inhibitor would be effective. Thrombin can be bound within the clot and only gradually released into the surrounding parenchyma. There may also be sources of thrombin other than from hematoma. There may be an influx of prothrombin into the brain due to the ICH-induced blood-brain barrier disruption. Thrombin may also be produced within the brain. Prothrombin mRNA is produced by brain cells and is upregulated after spinal cord injury.

It is a convention to express brain water contents as a percentage of tissue weight. This can sometimes be misleading. Thus, the contralateral and ipsilateral basal ganglia water contents after ICH were 78% and 82.6%. If it is assumed that the dry weight of the basal ganglia is unchanged, this apparently fairly small increase actually represents an increase from 3.55 to 4.75 g water per gram dry weight or a 26% increase in tissue mass (water+dry weight). Delayed administration of argatroban into the hematoma 3 hours after infusion blood reduced this swelling by approximately 40%. This experiment was designed to show that a delayed administration of argatroban could reduce edema formation if given directly to the clot, bypassing the need for the therapeutic agent to cross the blood-brain barrier. Such intracerebral injections have potential drawbacks as a therapeutic strategy, notably the delay enforced by the need for surgery and clot localization and the trauma associated with surgery. However, an intracerebral injection could have the potential advantage of allowing the argatroban to be infused away from the site of initial vascular rupture, perhaps limiting the chances of rebleeding.

Delayed systemic administration of argatroban (0.9 mg/h) begun 6 hours after infusion of blood also markedly reduced perihematomal edema, and it was approximately equally effective as an intracerebral injection. As shown by the results of stroke thrombolysis therapy, it is possible to admit and treat patients within a 6-hour therapeutic window. In animals in which systemic administration was delayed until 24 hours after the blood injection or a lower dose of argatroban was given (0.3 mg/h) at 6 hours, there was no reduction in perihematomal edema formation. Although the plasma argatroban concentration in the low-dose group (250 nmoL/L) exceeds the IC50 for thrombin inhibition by argatroban (30 nmoL/L), the brain concentration may have not exceeded the IC50.

It is difficult to translate animal data to humans. In these studies an intracerebral injection of 100 μL of blood was used. Expressed per gram of brain weight, this would correspond to an approximately 80-mL hematoma in humans. However, the absolute (rather than the relative) mass of the hematoma may affect the time window for argatroban treatment. In particular, the time course for release of thrombin from the hematoma to the surrounding brain may be prolonged if the hematoma mass is greater. Hamada and Matsuoka performed a small clinical trial with argatroban in ICH, starting argatroban infusion after 24 hours. In these few cases, perihematomal edema was less (and functional recovery was better), suggesting that the therapeutic window for argatroban may be longer in humans than in rats.

Argatroban was chosen as the thrombin inhibitor for these studies for a number of reasons. Most importantly, it is able to inhibit both soluble and clot-bound thrombin, and the latter may be particularly important in relation to its effects in ICH. The ability of argatroban to inhibit clot-bound thrombin is probably a reflection of its low molecular weight compared with hirudin and heparin. Its low molecular weight may also aid in crossing of the disrupted blood-brain barrier. In addition, unlike heparin, no cofactor is required for argatroban to inhibit thrombin. Additionally, argatroban does not directly affect thrombolytic enzymes, and it has a lower hemorrhagic potential in vivo than heparin or hirudin.

Although the present study focuses on thrombin-mediated edema formation after ICH, a number of events appear to contribute to edema at different times after ICH. These include a very early phase (first several hours) involving hydrostatic pressure and clot retraction, a second phase (first day) involving clotting cascade and thrombin production, and a third phase (after approximately 3 days) involving erythrocyte lysis and hemoglobin-induced toxicity and the potential generation of free radicals. Thus, although argatroban is successful in reducing ICH-induced edema, it is possible that the effectiveness of argatroban would be increased by combining it with an agent that reduces one of these other events (eg, hemoglobin-induced toxicity).

**Safety**

The primary ICH model used in this study involved direct injection of autologous blood into the caudate, and it did not have a disrupted vasculature as the basis of the ICH. To assess whether argatroban might induce rebleeding, the collagenase ICH model of Rosenberg et al was used. Although argatroban does not directly affect the fibrinolytic enzymes, it may exacerbate rebleeding by inhibiting clotting via thrombin inhibition and by reducing platelet adhesion to the vasculature. In the collagenase model there was no evidence of rebleeding after argatroban administration (systemic or intracerebral) as assessed by measurement of hematoma volume or brain hemoglobin concentration. The doses used were the same as those used to reduce edema formation. Spectrophotometric measurement of brain hemoglobin was used as well as direct measurement of hematoma volume because the effects of argatroban on thrombin might affect clot retraction and its effects on brain water content might also secondarily affect hematoma volume.

It is difficult to translate safety data between rats and humans. The rat collagenase model has a disrupted vasculature as the basis for the ICH, but the form of that disruption is different from a normal human ICH. Although most patients stop bleeding shortly after a spontaneous ICH, spontaneous rebleeding may occur. Most repeated hemorrhaging occurs within the first 24 hours. Fuji et al reported early expansion of the hematoma in 14% of patients within 24 hours and suggested that early hematoma growth is related to coagulation abnormalities such as platelet aggregation and low levels of fibrinogen. Brett et al demonstrated that 26% of patients had hematoma enlargement within the first hour and 38% had clot expansion within 20 hours. In view of this potential for spontaneous rebleeding, great care would be needed in determining the safety window for argatroban in humans.
Traumatic and Other Forms of Brain Injury

Two sets of experiments examined whether argatroban has an effect on brain water contents in the absence of ICH. In animals in which a needle was placed and 100 µL saline was infused, there was a small but significant increase in the ipsilateral basal ganglia water content compared with collateral. That edema was significantly reduced in the argatroban-treated group, although the degree of reduction was not sufficient to account for the reduction in ICH-induced edema with argatroban. However, these results support the concept that thrombin inhibition may decrease a variety of forms of brain injury. Thus, intraparenchymal argatroban reduces secondary brain injury that follows excision of part of the cortex, and systemic argatroban ameliorates ischemic brain injury.

Whether the protection in ischemia reflects purely vascular effects or some parenchymal actions as well merits further investigation.

In conclusion, these results demonstrate that the thrombin inhibitor argatroban can significantly reduce ICH-induced edema formation even when administration is delayed for 6 hours after ICH. In the rat, delayed administration of argatroban was not associated with an increase in rebleeding after ICH, suggesting that it may be a potential therapeutic agent for ICH-induced edema in humans.

Acknowledgment

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References

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