Mechanisms of edema formation after intracerebral hemorrhage: effects of thrombin on cerebral blood flow, blood-brain barrier permeability, and cell survival in a rat model

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Understanding the effects of an intracerebral hematoma on brain physiology is critical to developing rational management protocols. Both spontaneous and traumatic hematomas result in surrounding cell injury and brain edema. Increasing amounts of edema following intracerebral hemorrhage often result in worsening neurological function and brain herniation. Preventing the accumulation of edema fluid is therefore an important aspect of management.

The specific mechanism for brain edema formation that follows intracerebral hemorrhage has not been determined. The current study examines whether the action of thrombin is due to an effect on cerebral blood flow (CBF), vasoreactivity, blood-brain barrier (BBB) function, or cell viability. In vivo solutions of thrombin were infused stereotactically into the right basal ganglia of rats. The animals were sacrificed 24 hours later; CBF and BBB permeability were measured. The actions of thrombin on vasoreactivity were examined in vitro by superfusing thrombin on cortical brain slices while monitoring microvessel diameter with videomicroscopy. In separate experiments C6 glioma cells were exposed to various concentrations of thrombin, and lactate dehydrogenase release, a marker of cell death, was measured. The results indicate that thrombin induces BBB disruption as well as death of parenchymal cells, whereas CBF and vasoreactivity are not altered. The authors conclude that cell toxicity and BBB disruption by thrombin are triggering mechanisms for the edema formation that follows intracerebral hemorrhage.

KEY WORDS • intracerebral hemorrhage • thrombin • blood-brain barrier permeability • cerebral blood flow • C6 glioma cells • brain edema • rat
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likely attenuated by thrombin-specific inhibitors. Activation of the coagulation cascade with release of thrombin during clotting, therefore, appears to be the triggering event that initiates formation of brain edema.

Our research has shown that brain edema forms in response to thrombin even without fibrin clot formation. Fibrinogen, which is converted to fibrin clot through the enzymatic action of thrombin, does not affect the amount of edema produced by thrombin; therefore, the mechanism of edema formation does not involve the release of fibrinopeptides or fibrin degradation products. We have also shown that whole blood produces more brain edema than plasma or red blood cells alone. Prothrombinase, the enzyme complex that produces thrombin, has cofactors located in both the plasma component and the cellular component of blood. Factor V, factor X, and calcium from plasma, as well as phospholipids from cell membranes, all serve as cofactors that individually accelerate the conversion of prothrombin to thrombin. In whole blood, each of the cofactors is present; therefore, thrombin production and brain edema formation are maximal.

The pathophysiological effects of thrombin that account for brain injury and edema formation have not been elucidated. Brain edema from ICH and thrombin release may be due to ischemia, opening of the BBB, or direct toxicity to brain cells. In this study, CBF, microvessel diameter, BBB permeability, and brain cell survival were measured in tissue exposed to concentrations of thrombin consistent with intracerebral hemorrhage to clarify the events surrounding edema formation in the brain following ICH.

Materials and Methods

Animal Preparation

A total of 81 male Sprague–Dawley rats, each weighing between 250 and 350 g, were used in experiments of CBF and BBB permeability. The rats were anesthetized in a closed chamber with 5% isoflurane. A catheter was inserted into the trachea by the oral route. The rats were anesthetized in a closed chamber with 5% isoflurane and ventilated as previously described. Catheters were inserted into the right femoral artery and vein. Body temperature was maintained at 37.5°C using a rectal thermometer and a feedback-controlled heating pad.

Experimental Model

The rat was positioned in a stereotactic frame and the scalp was incised along the sagittal midline. An operating microscope was used for the remainder of the procedure, and hemostasis was maintained with bipolar electrocautery. A 1-mm burr hole was placed in the skull near the right coronal suture 3 mm lateral to the midline. A 26-gauge needle was inserted into the right basal ganglia under the microscope from t = 0 to t = T; and Qs(T) = quantity of indicator flow (milliliter/100 g/minute) for the brain samples was calculated using the equation: Fb/Mb = Qb(T)/Fs(Qs(T)*Mb*100, where Fb = cerebral blood flow; Mb = brain mass; Qb(T) = quantity of indicator present in the tissue at time T; Fs = rate of blood withdrawal into the syringe from t = 0 to t = T; and Qs(T) = quantity of indicator present in the cannula at time T.

Microvessel Diameter

In vitro brain slices were obtained from the rats and examined using computerized videomicroscopy with the methods described previously. Briefly, rats were anesthetized and perfused with 50 ml buffered saline followed by 10 ml 0.05% indigo carmine in saline. Each animal was decapitated and its brain was rapidly removed. Brain slices were obtained at a thickness of 300 to 500 μm with a tissue chopper and placed in artificial cerebrospinal fluid (CSF) (NaCl 124.1 mM, NaHCO₃ 25.7 mM, KCl 3.3 mM, KHPO₄ 1.3 mM, MgSO₄ 2.4 mM, CaCl₂ 2 mM, glucose 10 mM) in a holding chamber oxygenated with moist 95% O₂/5% CO₂ at 30°C.

Individual slices were mounted in a recording chamber on a transmission microscope and superfused at a constant rate with artificial CSF at 32°C. While in the recording chamber a vessel was selected and monitored continuously with a light microscope equipped with a CCD camera and a digital image analysis system. Microvessel diameter within the slice was monitored for 10 minutes while the slides were superfused with artificial CSF. Thrombin in artificial CSF was then allowed to superfuse the slices for 50 minutes while the microvessel diameter was monitored.

Because thrombin has been shown to cause release of endothelin, which has an immediate vasoconstrictive effect, we were interested in rapid vascular changes due to endothelin or the direct effects on the vascular smooth-muscle cells. An observation period of 1 hour was therefore adequate for these recordings. Previous studies using this system have shown that vascular responsiveness is retained in the brain slice preparation, as evidenced by constriction of microvessels due to potassium, endothelin, and phorbol dibutyrate. As a positive control for vasoconstriction in this study, microvessels were treated with endothelin-1 (100 nM) at the end of each experiment.

Blood-Brain Barrier Permeability

Blood-brain barrier permeability was measured in vivo at 24
hours after intracerebral infusion of thrombin or saline using an amino acid analog, α-aminoisobutyric acid (AIB), which is ordinarily not transported into the brain. The method has been previously described.2 A radioisotope of inulin, a molecule that remains intravascular despite breakdown of the BBB, was used to measure intravascular volume.

The rats that had received intracerebral infusions of thrombin or saline 24 hours earlier were anesthetized with ketamine (10 mg/kg) and xylazine (50 mg/kg), and catheters were placed in both femoral arteries and one femoral vein.

The rats received a bolus injection of 14C-AIB (25 µCi), and arterial blood was withdrawn at a constant rate (1 ml/minute) using a peristaltic pump. Eight minutes later the rats received an intravenous injection of 14C-inulin (15 µCi), which was allowed to circulate for 2 minutes before a terminal arterial sample was obtained and the rats were decapitated. The brain and arterial samples were processed as described for the CBF measurements, and the radioisotope content of both blood and tissue samples was determined using a two-channel scintillation counter.

For compounds of low permeability such as AIB, the influx rate constant (K1) can be calculated as: K1 = Cev(T)/Cad, where Cev(T) is the extravascular concentration of isotope at time T and Cad is the integral of the arterial tracer concentration from time 0 to time T. The Cev(T) was calculated from the total tracer counts Ctot(T) in brain samples, final tracer plasma concentration Cpl(T) and the plasma volume PV: Cev(T) = Ctot(T)/PV*Cpl(T).

Lactate Dehydrogenase Assay

Lactate dehydrogenase concentrations were measured using a commercially available diagnostic kit. One milliliter of 0.75 mmol/L sodium pyruvate was pipetted into a vial containing 1 mg β-nicotinamide adenine dinucleotide (1.28 µmol) and placed in a water bath at 37°C for 5 minutes. Fifty microliters of the sample was then added. Exactly 30 minutes after adding the sample, the vials were removed from the water bath and placed at room temperature. One milliliter of 2,4-dinitrophenylhydrazine (20 mg/dL) in 1 N hydrochloric acid was added. Twenty minutes later, 10 mL of 0.40 N sodium hydroxide was added to each vial. The samples were read against water at 450 nm with a spectrophotometer. A standard curve was prepared using various concentrations of sodium pyruvate corresponding to a range of LDH activities.

Statistical Analysis

Differences between groups of rats were evaluated using analysis of variance and the Dunnett t-test or Student t-test where indicated. A probability value of less than 0.05 was used to indicate a significant difference.

Cerebral Blood Flow

The physiological parameters recorded during intracerebral infusion are shown in Table 1. Blood pressure, blood gas levels, and hematocrit values all remained within normal ranges.

Figure 1 demonstrates CBF over time in animals receiving an intracerebral injection of 10 µl thrombin (10 U). This quantity of thrombin was shown in previous studies to produce brain edema and is equivalent to the amount produced by a moderate-sized hematoma.14,15 In the right hemisphere, left hemisphere, and cerebellum, blood flow declined over the 1st hour, rose to baseline or above by 2 hours, and then returned to baseline by 24 hours. The low-
The highest blood flow recorded was 44.6 ± 6.7 ml/100 g/minute in the right hemisphere at 1 hour.

Figure 2 compares CBF among rats receiving an intracerebral injection of thrombin and groups receiving saline at four time points. There were no differences between these groups at any time point. The reduction in blood flow seen with thrombin infusion was also documented in the saline-infused animals. Because the reduction in CBF was similar between thrombin and saline groups no vasoactive role could be attributed to thrombin alone. Instead, it appears that the mechanical trauma from needle placement and infusion of solution resulted in a decrease in CBF that lasted approximately 1 to 2 hours.

**Microvessel Diameter**

Microvessel diameter was measured from in vitro brain slices superfused with thrombin. Experiments were performed using 0.1, 100, and 1000 U/ml of thrombin in the perfusate. Figure 3 shows the results at 1000 U/ml. Similar results were seen at the other concentrations of thrombin. There was no change in vessel size with exposure of the vessels to thrombin as compared to the baseline values recorded during perfusion of the microscope slide with artificial CSF.

Endothelin-1, however, elicited a potent vasoconstrictor response in all microvessels (76.3 ± 4.2% constriction; mean ± standard error of the mean), showing that the vasoconstrictive capacity was preserved in these microvessel preparations.

**Blood-Brain Barrier Permeability**

Figure 4 shows the increase in BBB permeability for AIB, which is a marker of BBB opening. The PS product (a factor proportional to BBB permeability and capillary vessel surface area) was calculated for each region of the brain. In these experiments vessel surface area was assumed to remain constant. The differences in PS product between the right and left sides in each rat were used to determine the increase in permeability.

Saline infusion caused a slight increase in AIB influx (0.48 ± 0.06 µl/g/minute), but thrombin infusion caused a much larger increase (1.26 ± 0.19 µl/g/minute). These injections did not significantly affect the cerebellum. These values, therefore, indicate disruption of the BBB in the ipsilateral hemisphere due to thrombin.

**Brain Cell Toxicity**

Cell culture experiments were performed to determine whether thrombin has a direct toxic effect on brain cells. Figure 5 demonstrates the amount of LDH present in solutions containing C6 glioma cells exposed to various doses of thrombin for 24 hours. Because LDH is released from cells undergoing lysis, it is a marker of cell death. At a dose of 100 U/ml thrombin there was an increase in LDH at 24 hours, indicating a significant increase in cell death.

**Discussion**

Cushing believed that brain injury from ICH was due to local pressure compressing the microcirculation and producing ischemia in the region around a hematoma. Experimental studies have demonstrated CBF adjacent to an intracerebral hematoma does actually decrease. Cerebral blood flow in the rat falls below 25 ml/100 g/minute immediately adjacent to a hematoma. The decline in CBF, however, resolves within 10 minutes. The development of cerebral infarction is a function of both the degree and duration of ischemia; however, there is no experimental evidence that the degree or duration of hypoperfusion around an intracerebral hematoma is sufficient to produce ischemic cell damage. In monkeys, CBF below 10 to 12 ml/100 g/minute results in stroke only if sustained for 2 to 3 hours. Blood flow greater than 23 ml/100 g/minute does not produce infarction, even with permanent
ischemia. Thus, critical levels and durations of hypoperfusion have not been demonstrated immediately adjacent to a hematoma.

A global effect of the hematoma on CBF similarly does not explain the injury produced by most hematomas. An average-sized hematoma in humans (54 ml) has little immediate effect on intracranial pressure or cerebral perfusion pressure because that volume is well within the volume-buffering capacity of the intracranial space.\textsuperscript{10,20} Ischemia, therefore, is not initially the result of a global decline in perfusion pressure unless the mass is substantially greater than average.

There is evidence that intracerebral blood causes brain injury through biochemical substances released from the hematoma that initiate formation of brain edema.\textsuperscript{16,17} Edema from an average-sized clot is mostly due to these biochemical substances and not to mass effect.\textsuperscript{16,25} Recent studies of acute subdural hematomas have also implicated, but not identified, mediators of brain injury released from hematomas.\textsuperscript{12,13}

Edema may be a marker of the severity of the initial brain injury as well as a contributor to subsequent injury. There is an increase in brain edema after ICH over the first 24 hours in rats receiving injections of 100 μl of autologous blood into the basal ganglia.\textsuperscript{32} Brain edema reaches a peak level and plateaus during the first 4 to 5 days after exposure of the brain to blood. Edema then begins to resolve. Brain ions, such as sodium and chloride, increase for 3 days and then begin to return to baseline levels. Potassium content decreases over 3 days due to its release from brain cells. The increase in total brain ions accounts for the majority of the osmolar force driving water into the brain.\textsuperscript{32}

Thrombin, in amounts produced by clotted whole blood, causes changes in brain water and ion contents consistent with edema from an intracerebral hematoma.\textsuperscript{14,15} Furthermore, the brain edema and ion changes due to intracerebral blood have been shown to be inhibited by thrombin inhibitors.

Our initial model for brain injury after ICH can be summarized as follows. Hemorrhage into the brain activates the coagulation cascade, which produces a large quantity of thrombin that, in turn, activates pathways that produce brain edema. Inactivation of thrombin by antithrombin III and other plasma inhibitors regulates brain edema. In addition, edema formation is attenuated by protease nexin-1, a thrombin inhibitor unique to the brain. The results of the current study further elucidate the mechanism of brain edema formation due to blood.

Throughout these studies the thrombin dose represented the amount of thrombin produced by a moderate-sized intracerebral hematoma. Because an average-sized intracerebral hematoma in humans contains approximately 10,000 U prothrombin,\textsuperscript{34} a proportional hematoma in rats (50 μl) has the potential to produce 10 U thrombin. Ischemia from inadequate CBF has not been shown to contribute to brain injury from either blood or thrombin in our model. Blood flow in the cerebral hemisphere was reduced to 50% of control at 1 hour after placement of a hematoma.\textsuperscript{32} This level of flow was not below the ischemic threshold.\textsuperscript{8} Cerebral blood flow returns to baseline by 4 hours, where it remains for the next 24 hours. No period of significant hypoperfusion was observed in these intracerebral hematoma experiments. Cerebral blood flow also decreased in the remaining brain, implicating a global mechanism of blood flow regulation in response to injury. Similar diffuse declines in CBF have also been shown in patients.

A similar blood flow time course was seen with thrombin infusion. Like blood, thrombin infusion did not produce periods of critically low CBF. In the ipsilateral hemisphere, CBF decreased approximately 50% over the 1st hour, never falling below the ischemic threshold. Within 2 hours of thrombin infusion, blood flow returned to baseline and maintained that level over the next 24 hours.

There were no differences in CBF between animals in the thrombin group and animals in the control group at multiple time points after infusion. The decline in CBF over the 1st hour after intracerebral infusion of blood, thrombin, and saline was likely due to the mechanical trauma of needle insertion and fluid infusion. A decrease in CBF with mechanical trauma is probably a homeostat-
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![Bar graph comparing LDH release from C6 glioma cells in cultures exposed to various concentrations of thrombin. The values shown are means ± standard errors of the means in four experiments for each thrombin concentration. * = p < 0.01, control versus thrombin. The Dunnett t-test was used for comparison.](image)

Fig. 5. Bar graph comparing LDH release from C6 glioma cells in cultures exposed to various concentrations of thrombin. The values shown are means ± standard errors of the means in four experiments for each thrombin concentration. * = p < 0.01, control versus thrombin. The Dunnett t-test was used for comparison.

There is an increase in cerebral metabolism with a simultaneous reduction in regional CBF adjacent to a hematoma, indicating uncoupled flow and metabolism. This harmful effect of blood is mediated through N-methyl-D-aspartate receptors. Ischemic cell damage due to intracranial blood can be inhibited by NMDA antagonists. High concentrations of excitatory amino acids may exacerbate ischemic damage through an "excitotoxic" mechanism. This direct cell toxicity may be mediated by the large amount of thrombin released from hematomas. The primary event leading to brain injury and edema following ICH, therefore, appears to be blood-induced metabolic disruption initiated by a substance such as thrombin that diffuses from the hematoma into the brain. A metabolic insult leads to ischemic necrosis, even in the presence of CBF levels that are not below the usual ischemic threshold.

Conclusions

Brain injury and edema from an intracerebral hematoma appear to be due to thrombin that is produced by the coagulation cascade. Although some degree of reduction in local CBF occurs, ischemic levels are not present. Early edema is due to direct cell toxicity, possibly mediated through a cytotoxic receptor or zymogen activated by thrombin. An excitotoxic pathway may lead to cell injury. Later, breakdown of the BBB contributes to edema formation.

References

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