Effects of Thrombin on Neurogenesis After Intracerebral Hemorrhage

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Background and Purposes—Neurogenesis in intracerebral hemorrhage (ICH) has not been investigated. Thrombin formation causes acute brain injury after ICH, but thrombin also can stimulate cell proliferation. The present study examined whether neurogenesis takes place in ICH and the role of thrombin in ICH-related neurogenesis.

Methods—This study was divided into four parts. (1) Rats received either an ICH or a needle insertion (sham). The rats were killed for doublecortin (DCX) Western blot analysis and immunohistochemistry. (2) Rats had an ICH or a sham operation, and then received intraperitoneal injections of 5-bromo-2'-deoxyuridine (BrdU) at day-7 and day-9 later. Brains were perfused to identify BrdU-positive cells. (3) Rats had an intracaudate injection of thrombin (1 U) and brains were sampled for Western blots. (4) Rats had an ICH with or without a thrombin inhibitor, hirudin. The brains were sampled for DCX quantitation.

Results—DCX levels in the ipsilateral basal ganglia started to increase as early as 7 days after ICH, peaked at 14 days, and then gradually decreased at 1 month. Immunohistochemistry also demonstrated that DCX immunoreactivity was increased in the ipsilateral subventricular zone and basal ganglia at 2 weeks after ICH. Some DCX-positive cells were BrdU-positive. One unit thrombin, which does not cause marked brain injury, was injected into the caudate. Thrombin increased DCX levels in the ipsilateral basal ganglia and hirudin blocked ICH-induced upregulation of DCX.

Conclusions—Our results demonstrated that neurogenesis occurs in the brain after ICH and that thrombin may play a role in ICH-induced neurogenesis. (Stroke. 2008;39:2079-2084.)

Key Words: doublecortin ■ cerebral hemorrhage ■ neurogenesis ■ rat ■ thrombin

About 60 000 people in the United States suffer an intracerebral hemorrhage (ICH) each year. Of these 50% will die and a further 30% to 40% will suffer severe neurological impairment.1,2 There is currently no proven therapy for ICH other than supportive care.

Neurogenesis is modulated by both physiological stimuli and pathological conditions, where it may contribute to brain recovery. Neurogenesis has been well studied in ischemic stroke,3,4 but neurogenesis in ICH has not been well investigated although the delayed appearance of nestin-positive neuron-like cells around the lesion in a rat ICH model suggests that it might occur.5 Neurogenesis has been found after intracerebral injection of collagenase.6 Cell proliferation marker, bromodeoxyuridine (BrdU), and immature neuronal marker, doublecortin (DCX), are frequently used in neurogenesis studies.7

Our previous studies have demonstrated that thrombin has a key role in ICH-induced brain damage. Although high concentrations of thrombin cause brain edema and cell death, low concentrations are neuroprotective.8–10 A recent study showed that thrombin and thrombin receptor have a role in differentiation of progenitor cells.11 Thrombin also can stimulate cell proliferation.12–14 In addition, vascular endothelial growth factor (VEGF) plays an important role in neurogenesis,15,16 and thrombin can upregulate cell VEGF levels.17

The present study examined whether neurogenesis takes place in the rat blood injection ICH model and the effects thrombin on ICH-related neurogenesis.

Materials and Methods

Animal Preparation and ICH Model

Adult male Sprague-Dawley rats (Charles River Laboratories) were used for this study. Animal protocols were approved by the University of Michigan Committee on the Use and Care of Animals. One hundred six rats weighing 300 to 350 g were allowed free access to food and water. The animals were anesthetized with pentobarbital (45 mg/kg i.p.), 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. Rectal temperature was maintained at 37.5°C using a feedback-controlled heating pad. The rats were positioned in a stereotaxic frame (Kopf Instruments), and 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). Autologous whole blood with or

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without hirudin, and thrombin were injected at a rate of 10 μL/min with the use of a microinfusion pump (Harvard Apparatus Inc). The needle was removed, and the skin incision was closed with suture after infusion. For sham operation, procedure was the same, except for inserting the needle but no injection of blood.

Experimental Groups
This study was divided into 4 parts. In the first part, rats (n=3 to 6 each time point) received either an intracaudate injection of 100-μL autologous whole blood (ICH) or a needle insertion (sham). The rats were killed 1, 3, 7, 14, 30, 60, 90, and 180 days later for Western blot analysis and immunohistochemistry of doublecortin (DCX). In the second part, rats (n=3 to 4 each group) had an ICH or a sham operation and then received intraperitoneal injections of 5-bromo-2’-deoxyuridine (BrdU, 50 mg/kg) at day 7 and day 9 after ICH or sham operation. Brains were perfused at 14 days after ICH to identify BrdU-positive cells. In the third part, rats (n=3 to 4 each group) had an intracaudate injection of 50-μL thrombin (1 U), and brains were sampled for Western blots 3, 7, 14, and 30 days later. In the last part, rats (n=3 to 4 each group) received an injection of 100-μL blood with or without a thrombin inhibitor, hirudin (5 U). The brains were sampled at 14 days for DCX quantitation.

BrdU Labeling
The thymidine analog BrdU (Sigma) was used to label S-phase cells. Rats received BrdU injections intraperitoneally, 50 mg/kg in phosphate-buffered saline, 2 times a day, on day 7 to day 9 after ICH or sham operation.

Western Blot Analysis
Animals were anesthetized before undergoing intracardiac perfusion with saline. The brains were then removed and a 3-mm-thick coronal brain slice was cut approximately 4 mm from the frontal pole. The slice was separated into ipsilateral and contralateral basal ganglia. Western blot analysis was performed as previously described. Briefly, 50 μg proteins for each were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a Hybond-C pure nitrocellulose membrane (Amersham). The membranes were blocked in Carnation nonfat milk. Membranes were probed with a 1:1500 dilution of the primary antibody (goat anti-DCX, Santa Cruz Biotech) and a 1:2000 dilution of the secondary antibody (peroxoxygenase-conjugated Rabbit antigen antibody, Jackson ImmunoResearch Laboratories Inc). The antigen-antibody complexes were visualized with a chemiluminescence system (Amersham) and exposed to Kodak X-OMAT film. The relative densities of bands (45 kDa) were analyzed with NIH Image (Version 1.61).

Immunofluorescent Double Labeling and Confocal Microscopy
For immunofluorescent double labeling, primary antibodies were goat anti-DCX and mouse anti-BrdU. Rhodamine conjugated rabbit antibody (1:100) and fluorescein isothiocyanate (FITC) labeled horse antimouse (1:100) second antibodies were incubated in 1:10 rabbit or horse serum for 30 minutes, rinsed, and incubated overnight with the primary antibody. The primary antibody was polyclonal goat anti-DCX (Santa Cruz Biotech). Normal goat IgG was used as a negative control. Sections were incubated with 1:1000 dilution of biotinylated rabbit antibody IgG (Vector Laboratories) for 90 minutes and then incubated with avidin-biotinylated horseradish peroxidase (Vector Laboratories) for 90 minutes.

Statistical Analysis
All data in this study are presented as mean±SD. Data were analyzed with Student t test and analysis of variance (ANOVA), followed by Scheffe post hoc test. Significance levels were measured at P<0.05.

Results
All physiological variables were measured immediately before intracerebral infusions. Mean arterial blood pressure (MABP), pH, arterial oxygen and carbon dioxide tensions (pO2 and pCO2), hematocrit, and blood glucose were controlled with normal range (MABP, 80 to 120 mm Hg; pO2, 80 to
120 mm Hg; pCO2, 35 to 45 mm Hg; hematocrit, 38 to 43%; blood glucose, 80 to 120 mg/dL).

The time course of DCX expression was examined by Western blot analysis. DCX levels in the ipsilateral basal ganglia started to increase as early as 7 days after ICH (170±49% of sham, Figure 1), peaked at 14 days (805±241% of sham, P<0.01), and then gradually decreased at 1 (437±161%, P<0.05) and 2 months (360±104%, P<0.05, Figure 1). At 2 weeks after ICH, DCX protein levels in the ipsilateral basal ganglia were strongly increased.

Figure 3. DCX immunoreactivity around the subventricular zone (SVZ) and the basal ganglia (BG) 14 days after an ICH or sham operation. A, D, G, Contralateral to ICH; B, E, H, Ipsilateral to ICH; C, F, I, Ipsilateral to sham operation. * indicates the site for high power images at the subventricular zone (D, E, F). ** indicates the site for high power images of the basal ganglia (G, H, I). Scale bar=200 μm (A-C) and 20 μm (D-I).

Figure 4. Immunohistochemistry showing 5’-bromodeoxyuridine (BrdU) immunoreactivity in the contralateral (A) and ipsilateral (B) subventricular zone 14 days after ICH. Scale bar=100 μm. C, Immunofluorescent double labeling showing colocalization of BrdU and DCX in the ipsilateral subventricular zone 14 days after ICH. Scale bar=20 μm.
Immunohistochemistry also demonstrated that DCX immunoreactivity was markedly increased in the ipsilateral subventricular zone and basal ganglia at 2 weeks after ICH (Figure 3). Figure 3H showed DCX-positive cells migrating to injured basal ganglia after ICH. No immunoreactivity was found in the brain sections of negative control (data not shown).

After ICH, BrdU-positive cells were more prevalent in the ipsilateral subventricular zone compared to the contralateral side (Figure 4). To confirm whether the neuroblasts were newly generated after ICH, we performed immunofluorescence double staining for DCX and BrdU. We found that DCX-positive cells were BrdU-positive (Figure 4C).

To test the role of thrombin in neurogenesis, 1 unit thrombin, which does not cause marked brain injury, was injected into the caudate. Intracaudate injection of thrombin increased DCX levels in the ipsilateral basal ganglia at day 7 ($P<0.05$, Figure 5).

Hirudin is a specific thrombin inhibitor. Hirudin blocked ICH-induced upregulation of DCX in the ipsilateral basal ganglia (151±94 versus 2689±837 pixels in the vehicletreated group, $P<0.05$; Figure 6).

**Discussion**

The major findings of the current study are that neurogenesis occurs over a prolonged period after ICH and that thrombin may be a key modulator in ICH-induced neurogenesis.

The role of neurogenesis in neurological diseases remains controversial. Studies have demonstrated the existence of progenitor cells and their potential for neurogenesis in the subventricular zone (SVZ), hippocampus dentate gyrus, and cortex of adult mammalian brain. Neurogenesis can be induced by various insults including cerebral ischemia. Neural regeneration may contribute to brain recovery after experimental cerebral ischemia. Neurogenesis also occurs in a mouse model of subarachnoid hemorrhage. Very recently neurogenesis has been found in a collagenase injection rat ICH model. Our present results show that neurogenesis also occurs in the blood injection ICH model and lasts for at least 6 months. In earlier studies, we have shown a marked recovery of function over the weeks after ICH. Temporally, there is some concordance between neurogenesis and this functional improvement. However, it is still uncertain as to whether or not ICH-induced neurogenesis contributes to functional recovery.

The importance of thrombin in modulating brain injury after stroke has become clear. Recent studies have demonstrated a role of thrombin and its receptors in progenitor cells. For example, thrombin stimulates differentiation of bone marrow–derived endothelial progenitor cells. In addition, thrombin enhances the synthesis and secretion of nerve growth factor in glial cells, modulates neurite outgrowth, and stimulates astrocyte proliferation.

The effects of thrombin on neurogenesis may, at least in part, be through activation of thrombin receptors. Thrombin receptors are 7 transmembrane G protein–coupled receptors that are activated by proteolytic cleavage. Three protease-activated receptors (PARs), PAR-1, PAR-3, and PAR-4, can be activated by thrombin. PAR-1 expression is found in neurons, astrocytes, oligodendroglial cells, and microglia, and there is functional evidence for the presence of PAR-1 on all cell types. PAR-1 may be the main thrombin receptor mediating thrombin-related neurogenesis. For instance, PAR-1 activation in human endothelial progenitor cells modulates the angiopoietin pathway and has been associated with angiogenesis. PAR-1 activation also stimulates progenitor cell differentiation. Future studies should investigate the effects of PAR-1 activation on ICH-induced neurogenesis.

Many factors, including vascular endothelial growth factor (VEGF), may modulate neurogenesis after stroke. VEGF is a specific mitogen of endothelial cells and a strong stimulator of angiogenesis, but recent studies have also found a significant role of VEGF in neurogenesis. Formation of a neurovascular niche may be important for neurogenesis after brain damage. Although thrombin can stimulate cells to produce VEGF, it is unclear whether or not thrombin-related neurogenesis in ICH is partially VEGF mediated.
BrdU and DCX are 2 markers often used in neurogenesis studies. BrdU labels cells in S-phase. Recent studies, however, have shown that apoptotic cells can also be labeled with BrdU. Therefore, we also used DCX, a marker of new neurons, and colocalization with BrdU as assessed with confocal microscopy. We found no evidence of increased neurogenesis in our sham-operated rats that had just a needle insertion. Our previous studies have demonstrated that either needle insertion or needle insertion with saline injection results in minimal brain injury.

In summary, this study demonstrates that neurogenesis occurs after ICH. An intracerebral injection of thrombin induces neurogenesis, whereas thrombin inhibition reduces ICH-induced neurogenesis. These results suggest an important role of thrombin in neurogenesis after ICH.

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Disclosures
None.

References
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