Holo-Transferrin and Thrombin Can Interact to Cause Brain Damage

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Background and Purpose—Previous studies have suggested that delayed release of hemoglobin degradation products, particularly iron, is involved in intracerebral hemorrhage (ICH)-induced brain injury. However, a recent study found evidence of iron-induced brain injury soon after ICH. This study, therefore, examined whether another iron-containing component of blood, holo-transferrin (holo-Tf), might also induce brain injury either alone or in combination with thrombin, another factor involved in early ICH-induced brain injury.

Methods—Male Sprague-Dawley rats received an intracerebral infusion of holo-Tf, apo (noniron–loaded)-Tf, thrombin, or a combination of Tf with thrombin into the right basal ganglia. The rats were euthanized 24 hours later for measurement of brain edema and assessment of DNA damage (single- and double-strand breaks and 8-hydroxyl-2'-deoxyguanosine immunohistochemistry). Iron distribution was examined histochemically.

Results—Holo-Tf, apo-Tf, and the dose of thrombin used (1 U) all failed to induce brain edema when administered alone. However, the combination of holo-Tf with thrombin (but not apo-Tf with thrombin) caused brain edema, DNA damage, and intracellular iron accumulation in the ipsilateral basal ganglia.

Conclusions—These results suggest that in addition to hemoglobin-bound iron, Tf-bound iron may contribute to ICH-induced brain injury and that thrombin may contribute to the latter by facilitating cellular iron uptake. (Stroke. 2005;36:348-352.)

Key Words: brain edema ■ iron ■ oxidative stress ■ thrombin ■ transferrin

Animal models have implicated iron and thrombin in intracerebral hemorrhage (ICH)-induced brain injury.1–3 Thrombin is produced during clot formation, and intraparenchymal thrombin can induce acute brain injury.3,4 Thrombin inhibition reduces ICH-induced brain edema and behavioral deficits.5,6 Quantitatively, the major source of iron in the hematoma is erythrocyte hemoglobin. Intracerebral infusion of lysed erythrocytes causes acute brain injury, an effect mimicked by infusion of hemoglobin and its degradation product iron.7 In contrast, injection of packed erythrocytes causes delayed brain injury that is temporally related with erythrocyte lysis at ≈3 days.8 ICH induces oxidative brain damage9–11 and, because iron can cause free radical formation,12 we examined the effects of an iron chelator deferoxamine on ICH-induced brain injury in the rat. Interestingly, deferoxamine reduced acute brain injury and although it was effective when given 6 hours after ICH, it was not effective when administered after 24 hours.11 These results suggested that there might be a component of iron-mediated damage not related to hemoglobin breakdown and erythrocyte lysis. The current experiments, therefore, examined whether the iron bound to transferrin that would normally be present in plasma might be able to cause brain injury.

Iron-loaded (holo)-transferrin (Tf) or iron-depleted (apo-) Tf in the presence and absence of thrombin was administered intracerebrally to the rat. The effects on brain edema, DNA damage, oxidative stress, and iron distribution were examined.

Materials and Methods

Animal Preparation and Intracerebral Infusion

Animal protocols were approved by the University of Michigan Committee on the Use and Care of Animals. Forty-six male Sprague-Dawley rats (300 to 400 g; Charles River Laboratories, Wilmington, Mass) were used in the experiments. Rats were allowed free access to food and water. The animals were anesthetized with pentobarbital (40 mg/kg IP), and a 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 and maintained in the physiological range. 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 stereotactically into the right basal ganglia (coordinates:
0.2 mm anterior, 5.5 mm ventral, and 3.5 mm lateral to the bregma. Apo-Tf (0.4 mg; Sigma), holo-Tf (0.4 mg; Sigma), thrombin (1U; Sigma), apo-Tf + thrombin (0.4 mg and 1 U, respectively), or holo-Tf + thrombin (0.4 mg and 1 U, respectively) were dissolved in 50 μL saline and infused at a rate of 10 μL/min through a microinfusion pump (Harvard Apparatus). The needle was removed and the skin incision closed.

The dose of TF was based on normal plasma levels, which are ~3 to 5 mg/mL plasma. The percent of TF that is iron loaded (holo-TF) is dependent on iron status, but in rat it is normally 30% to 40%. The dose of thrombin was chosen for 2 reasons. First, this dose when administered alone does not induce brain injury; facilitating the examination of whether it might enhance holo-TF-induced brain injury. In addition, we found that intracerebral administration of 1U thrombin markedly upregulated expression of the TF receptor in brain, a potential point of interaction between thrombin and TF.

Experimental Groups
There were 3 sets of experiments in this proposal. In the first set, rats (n=5 per group) received an infusion of either apo-Tf, holo-Tf, thrombin, apo-Tf plus thrombin, or holo-Tf plus thrombin into the right basal ganglia. The rats were euthanized 24 hours later for brain water content measurement. In the second set, animals received an intracerebral infusion of either holo-Tf, apo-Tf plus thrombin, or holo-Tf plus thrombin. The brains (3 per group) were processed and used for 8-hydroxy-2′-deoxyguanosine (8-OHdG) immunohistochemistry, DNA polymerase I-mediated biotin-dATP nick translation (PANT) labeling, TUNEL, and iron histochemistry. In the third set, animals received an intracerebral infusion of either holo-Tf, apo-Tf plus thrombin, or holo-Tf plus thrombin or no infusion. The brains (3 per group) were processed for Western blotting of Ku70 and Ku86, 2 DNA repair proteins.

Brain Water Content
Animals were anesthetized (pentobarbital 50 mg/kg IP) and decapitated. The brains were removed, and a coronal brain slice (~3 mm thick) 4 mm from the frontal pole was cut. That slice was divided into the 2 hemispheres and each hemisphere dissected into cortex and basal ganglia. The cerebellum served as a control. Brain samples were immediately weighed on an electronic analytical balance to obtain wet weight before drying at 100°C for 24 hours to obtain dry weight. Percent water content was calculated as 100(wet weight−dry weight)/wet weight).

Immunohistochemistry and Histochemistry
Rats were anesthetized and underwent intracardiac perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). The brains were removed and kept in 4% paraformaldehyde for 12 hours, then immersed in 25% sucrose for 3 to 4 days at 4°C. Brains were then placed in embedding optimal cutting temperature compound (Sakura Finetek USA Inc) and sectioned on a cryostat (18 μm thick).

For 8-OHdG immunohistochemistry, the avidin-biotin complex technique was used. Sections were incubated in 1:10 horse serum (Vector Laboratories) for 30 minutes, rinsed, and incubated overnight with the primary antibody. The primary antibody was mouse anti-8-OHdG monoclonal antibody (10 μg/mL; Oxis International Inc; Portland, Ore). Normal mouse IgG (Vector Laboratories, Burlingame, Calif) was used as negative control. The second antibody was anti-mouse IgG antibody (1:150; Vector Laboratories, Burlingame, Calif).

DNA fragmentation was detected using methods described in Wu et al.; PANT assay was used to detect DNA single-strand breaks, and TUNEL technique was performed on adjacent brain sections to detect DNA double-strand breaks. For both methods, sections were permeabilized with 1% Triton X-100 for 30 minutes and quenched the endogenous peroxidases with 2% H2O2 for 20 minutes. For PANT staining, sections were then washed with PBS and incubated in a moist chamber at 37°C for 90 minutes with PANT reaction mixture. The sections were then incubated in streptavidin-horseradish peroxidase (Vetastain Elite ABC, Vector Laboratories) in PBS containing bovine serum albumin for 90 minutes at room temperature. Detection of the biotin-streptavidin-peroxidase complex was carried out by incubating sections with diaminobenzidine (DAB) solution (1:1, 3,3′-diaminobenzidine in PBS and 0.05% H2O2). Sections incubated with reaction mixture without DNA polymerase were used as control.

For TUNEL staining, an ApopTag Peroxidase Kit (Serologicals) was used. Permeabilized and quenched sections were washed with PBS and then incubated in a moist chamber at 37°C for 60 minutes with terminal deoxynucleotidyltransferase enzyme. After antidigoxigenin peroxidase had been applied to the sections for 30 minutes at room temperature, peroxidase was detected with DAB. The omission of the terminal deoxynucleotidyl transferase was used as the negative control.

For detection of ferric iron, a modified Perls’ staining was performed. Sections were washed with distilled water and incubated in Perls’ solution (1:1, 5% potassium ferrocyanide and 5% hydrochloric acid) for 45 minutes, followed by washing with distilled water 6X for 5 minutes each. The Perls’ stained sections were then incubated 0.5% DAB with nickel solution for 60 minutes.

Statistical Analysis
All data in this study are presented as mean±SD. Western blot and water content data were analyzed by ANOVA, followed by a Scheffe post hoc test for multiple comparisons. Values where P<0.05 were taken as significant.

Results

Brain Water Content
Intracerebral infusion of holo-Tf, apo-Tf, or 1U thrombin all failed to induce brain edema in the ipsilateral basal ganglia and cortex at 24 hours (Figure 1). In contrast, coadministration of holo-Tf with thrombin, but not apo-Tf with thrombin, resulted in marked edema in the ipsilateral basal ganglia and as well as ipsilateral cortical edema (Figure 1).

Iron Staining After Holo-Tf Infusion
To investigate the potential mechanisms involved in the brain injury after coadministration of holo-Tf and thrombin, Perls’ staining for iron was used. Injection of holo-Tf alone did not result in the appearance of Perls-positive cells (Figure 2a). In contrast, coadministration of holo-Tf and thrombin resulted in profuse Perls-positive cells in the ipsilateral but not the contralateral basal ganglia (Figure 2b and 2c). This effect of holo-Tf and thrombin was not mimicked by coadministration of apo-Tf and thrombin (Figure 2d).
DNA Damage and DNA Repair Proteins

To detect oxidative DNA damage, 8-OHdG immunohistochemistry was performed at 24 hours after intracerebral infusions. Similar to the results on iron distribution, 8-OHdG immunoreactivity was absent in the ipsilateral basal ganglia after injection of holo-Tf alone (Figure 3a) but was profuse in the ipsilateral but not the contralateral basal ganglia when holo-Tf was coinjected with thrombin (Figure 3b and 3c). 8-OHdG immunoreactivity was absent from the ipsilateral basal ganglia when apo-Tf rather than holo-Tf was coadministered with thrombin (Figure 3d).

To detect DNA fragmentation, TUNEL and PANT staining was performed at 24 hours after infusions. Again, similar to the results on iron distribution and 8-OHdG immunoreactivity, TUNEL-positive cells were absent in the ipsilateral basal ganglia after injection of holo-Tf alone (Figure 4a) but were numerous in the ipsilateral but not the contralateral basal ganglia when holo-Tf was coinjected with thrombin (Figure 4b and 4c). The TUNEL-positive cells in this group showed apoptotic characteristics, such as chromatin condensation and fragmented nuclei. TUNEL positive cells were absent from the ipsilateral basal ganglia when apo-Tf was coadministered with thrombin (Figure 4d).

PANT-positive cells were also present in the ipsilateral but not the contralateral basal ganglia of rats treated with holo-Tf and thrombin (Figure 4f and 4g). PANT-positive cells were absent in the ipsilateral basal ganglia of animals treated with holo-Tf alone or apo-Tf with thrombin (Figure 4e and 4h). Alterations in the levels of DNA repair proteins might contribute to DNA damage. In this study, there was a significant decrease in the protein levels of the DNA repair proteins Ku70 and Ku86 in the ipsilateral basal ganglia in rats treated with holo-Tf and thrombin compared with animals infused with holo-Tf alone or apo-Tf and thrombin (Figure 5a and 5b).

Discussion

This study shows that intracerebral coinjection of holo-Tf with thrombin causes brain edema, increased cellular iron levels, oxidative damage, and DNA fragmentation. These effects were not found in rats treated with holo-Tf alone or apo-Tf with thrombin. Given that we recently found that thrombin upregulates the Tf receptor on neurons, the results suggest a scenario whereby thrombin enhances the uptake of the iron from holo-Tf into brain cells causing increased intracellular iron, oxidative damage, DNA fragmentation, and cell injury. Thus, the presence of holo-Tf and thrombin during the formation of an intracerebral hematoma may participate in ICH-induced brain injury. This may explain why the iron chelator deferoxamine affects acute ICH-induced brain injury.

Transferrin is the main mechanism by which iron is transported between tissues in plasma. Transferrin exists in plasma in either the ferric iron–bound state (holo-Tf) or with no ferric ironbound (apo-Tf). Normally, approximately 30% to 40% is...
present as holo-Tf. Holo-Tf has high affinity for the Tf receptor compared with apo-Tf, and receptor interaction results in endocytosis and release of the reduced form of iron, ferrous iron, in the cell. In the brain, the Tf receptor is normally found at high levels in the endothelial cells forming the blood-brain barrier where it is involved in the transport of iron between blood and brain with relatively few receptors on most brain parenchymal cells. However, we recently found that intracerebral infusion of 1 U thrombin (as used in this study) causes a very marked upregulation in receptor levels in the ipsilateral basal ganglia at day 1 and day 3. ICH also increases brain Tf receptor levels. Unlike with infusion of holo-Tf alone, coadministration of holo-Tf and thrombin caused a marked increase in cellular iron staining. It appears very likely that an upregulation in brain Tf receptors levels by thrombin allows the uptake of the iron bound to transferrin into the parenchymal cells.

Increases in brain iron can result in lipid peroxidation and free radical formation. One target of free radicals is DNA.
and in this study infusion of holo-Tf with thrombin resulted in oxidative modification of DNA as assessed by 8-OHdG staining. This effect was not mimicked by infusion of holo-TF alone or apo-TF with thrombin. Oxidative DNA damage results in DNA fragmentation. In this study, DNA fragmentation was evident with holo-TF and thrombin coadministration as assessed by both PANT and TUNEL staining. Again TUNEL and PANT staining was absent in animals treated with holo-TF alone or apo-TF and thrombin.

Other studies have indicated that the uptake of iron bound Tf can result in free radical formation, oxidative damage, and cell death. Thus, Tf-receptor mediated iron uptake has been implicated in oxidative damage in endothelial cells and neurons. Interestingly, coadministration of holo-TF with thrombin was found to decrease the level of these proteins. Similar reductions have been found in cerebral ischemia/reperfusion injury. Two such proteins are Ku70 and Ku86. Interestingly, coadministration of holo-TF with thrombin was found to decrease the level of these proteins. Similar reductions have been found in cerebral ischemia/reperfusion injury. Two such proteins are Ku70 and Ku86.

Cells have DNA repair pathways that may limit DNA damage during oxidative stress. Two such proteins are Ku70 and Ku86. Interestingly, coadministration of holo-TF with thrombin was found to decrease the level of these proteins. Similar reductions have been found in cerebral ischemia/reperfusion injury. Two such proteins are Ku70 and Ku86.

The dose of thrombin used in this study (1 U), when administered intracerebrally alone, did not cause evident brain injury. Indeed, we have found that pretreatment with this dose of thrombin protects against a variety of brain injuries (thrombin preconditioning). In contrast, intracerebral infusion of higher doses (5 or 10 U) causes brain damage. However, under certain conditions injury can occur at low doses. Apart from this study, we have also found that injection of 1 unit of thrombin with tissue plasminogen activator during cerebral ischemia exacerbates injury. This suggests that thrombin has the potential to induce cell injury differently, dependent on dose and cellular environment. The extent to which enhanced iron uptake through the Tf receptor is involved in these different conditions has still to be determined. In particular, whether thrombin-induced brain injury during ICH is primarily mediated through iron has yet to be elucidated. Interestingly, Wagner et al recently reported that a plasma component is capable of rapidly inducing oxidative stress in white matter.

In summary, this study demonstrates that coadministration of holo-TF with thrombin causes marked brain injury that appears to be linked to thrombin-induced Tf receptor upregulation, enhanced iron uptake, and oxidative stress. This pathway may be an important early cause of brain injury after ICH and it may be amenable to therapeutic intervention.

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References

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