Hematoma resolution occurs in the days after intracerebral hemorrhage (ICH). The hematoma clearance may be via red blood cell (RBC) lysis or phagocytosis. Erythrocytes within the clot preserve a normal biconcave configuration for a while and then start to lyse. At the same time, erythrocytes are phagocytized by microglia/macrophages.

Erythrocyte lysis after ICH releases hemoglobin and its degradation products, such as iron, contributing to oxidative stress, delayed brain edema, and neuronal death. Erythrocyte lysis after ICH can be mediated by the complement activation and formation of the membrane attack complex (MAC), which contains complement C5b, C6, C7, C8, and C9 proteins (C5b–9). Our previous studies have demonstrated that complement depletion or complement inhibition reduces brain injury in a rat ICH model. These results indicate that membrane attack complex accumulation and decreased hemoglobin levels. Erythrophagocytosis occurred in the hematoma, and this was associated with reduced clot CD47 levels. Activated macrophages/microglia were CD163 and heme oxygenase-1 positive, and these accumulated in the clot with time. Deferoxamine treatment attenuated the process of hematoma resolution by reducing member attack complex formation and inhibiting CD47 loss in the clot.

The present study examined the process of hematoma clearance after ICH, which can be altered by deferoxamine treatment. (Stroke. 2016;47:1626-1631. DOI: 10.1161/STROKEAHA.116.013146.)

Key Words: cerebral hemorrhage • deferoxamine • hemolysis • membrane attack complex • swine

Materials and Methods

Animal Preparation and Intracerebral Blood Injection

Animal procedure protocols were approved by the University Committee on Use and Care of Animals, University of Michigan, and were conducted in accordance with United State Public Health Service’s Policy on Humane Care and Use of Laboratory Animals.
A total of 65 male piglets (8–10 kg; Michigan State University, East Lansing) were used in this study. One piglet was excluded because of high fever after surgery. The ICH models were performed as previously described. Pigs were sedated with ketamine (25 mg/kg, IM) and anesthetized with 2% isoflurane via nose cone. The isoflurane concentration was maintained at 1.0% to 1.5% during the surgical procedures. Core body temperature was maintained at 37.5±0.5°C by a feedback-controlled heating pad (Gaymar, Orchard Park, NY). The right femoral artery was inserted with a polyethylene catheter (PE-160) to obtain blood for injection and to monitor arterial blood pressure, blood gases, and glucose concentrations. A cranial burr hole (1.5 mm) was drilled at the point 11 mm right of the sagittal suture and 11 mm anterior to the coronal suture. An 18-mm-long 20-gauge sterile plastic catheter was placed stereotaxically into the center of the right frontal cerebral white matter at the level of the caudate nucleus.

First, 1.0 mL of autologous arterial blood was infused >10 minutes with an infusion pump. Then 1.5-mL blood was injected >10 minutes after a 5-minute break. After injection, the needle was removed, the burr hole was filled with bone wax, and the skin incision was closed with sutures.

Experimental Groups

There were 2 parts in this study. In the first part, piglets had ICH in the right frontal lobe. Brains were harvested at 4 hours or days 1, 3, and 7 (n=8 each group) for brain histology, ELISA, and Western blots. In the second part, piglets had ICH in the frontal lobe and were treated with vehicle (saline) or deferoxamine (50 mg/kg, IM at 2 hours after ICH and then every 12 hours). Brains were harvested at days 3 (n=8 each group) and 7 (n=8 each group) for brain histology, ELISA, and Western blots.

Brain In Situ Freezing and Brain Histology

Brains were frozen in situ by decanting liquid nitrogen into a 12 oz. bottomless foam cup adhered to the head with Dow Corning high vacuum grease (Dow Corning, Midland, MI), as described previously. The duration time of this process usually took >1 hour, and the head was removed after the injection of potassium chloride to stop the heart beating. The frozen head was then cut with a band saw into 5-mm-thick coronal sections. Tissues were sampled from the points of interest.

For brain histology, piglets were reanesthetized and the brains perfused in situ with 10% formalin. Brains were sectioned coronally. Paraffin-embedded brain was cut into 10-μm-thick sections.

ELISA

For hemoglobin and MAC measurement, the brains were in situ frozen, and hematoma tissue samples were collected and homogenized. The amount of hemoglobin and MAC was measured using Pig Hemoglobin ELISA kit (LifeSpan Biosciences, LS-F8543) and Pig C5b–9 Terminal Complement Complex ELISA kit (LifeSpan Biosciences, LS-F6300), according to the manufacturer’s instructions.

Hematoxylin and Eosin Staining and Measurement of RBC Diameter

Hematoxylin and eosin staining was used to examine RBC morphology and erythrocyte phagocytosis. Images were processed with Image J for measuring the diameter of RBC. The means were calculated from 30 RBCs per microscopic field in the hematoma edge and hematoma center, respectively, and 3 sections were analyzed for each brain.

Immunohistochemistry and Cell Counting

Immunohistochemistry staining was performed as described previously. Briefly, the sections were deparaffinized in xylene and rehydrated in a graded series of alcohol dilutions. Antigen retrieval was performed by the microwave method with citrate buffer (10 mmol/L, pH 6.0). Immunohistochemistry studies were performed with avidin–biotin complex techniques. The primary antibodies were polyclonal rabbit anti-HO-1 (Enzo, ADI-SPA-895-F, 1:400 dilution) and CD163 (AbD, MAC342R, 1:100 dilution). Counting of CD163-positive cells was performed on high-power images (~40 magnification) taken using a digital camera. Counts were performed on 4 areas in each brain section.

Western Blotting

Western blot analysis was performed as previously described. Briefly, brains were in situ frozen and the hematoma was sampled. Protein concentration was determined by Bio-Rad protein assay kit (Hercules, CA). An equal amount of protein (50 μg) was suspended in loading buffer, denatured at 95°C for 5 minutes and loaded on an SDS-PAGE gel. After being electrophoresed and transferred onto Hybond-C pure nitrocellulose membrane (Amersham), the membrane was blocked with nonfat milk buffer for 2 hours and then incubated with the primary antibodies. The primary antibodies were polyclonal rabbit anti-HO-1 (Enzo, ADI-SPA-895-F, 1:1000 dilution), monoclonal mouse antihuman CD47 (AbD, MCA911, 1:1000 dilution), and monoclonal mouse anti-GAPDH (Fitzgerald, 10R-G109A, 1:10 000 dilution). The membranes were incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature. Protein band densities were detected by Kodak X-OMAT film and quantified by NIH Image J. HO-1 and CD47 levels in the hematoma were expressed as the ratio of HO-1/GAPDH or CD47/GAPDH.

Statistical Analysis

Measurements were performed by personnel blinded to treatment groups. All the data are expressed as mean±SD. Data were analyzed by Student t tests or 1-way ANOVA. Differences were considered significant at P<0.05.

Results

The process of hematoma resolution was examined on coronal sections of pig brain after hematoxylin and eosin staining. Erythrocyte lysis occurs in the hematoma. To examine whether the morphology of RBC changed in the development of hemorrhage resolution, the diameters of RBC in the hematoma edge and hematoma center were measured. We found that diameter of RBC decreased gradually in the hematoma edge and hematoma center (eg, day 3 at hematoma edge: 2.71±0.38 versus 3.89±0.35 μm at 4 hours; P<0.01 and hematoma center: 2.61±0.29 versus 3.75±0.25 μm at 4 hours; P<0.01; Figure 1). The majority of RBCs at 4 hours after ICH were of double-faced concave disc shape (discocyte; Figure 1). RBCs in hematoma edge were fairly irregular in shape with spike at day 3 after ICH, whereas the RBC in center-hematoma deformed into uniformly sized spherical cells (spherocyte; Figure 1). The transformation of erythrocyte shape from discocyte to spherocyte might be because of changes in membrane permeability.

Hemoglobin contents in the clot (Figure 2A) were measured to assess hematoma resolution. The level of hemoglobin declined gradually over the time after the onset of ICH with a significant reduction at day 3. The contents of hemoglobin in the clot were 26.2±5.2 mg/g at 4 hours but were reduced to 13.9±0.9 mg/g at day 3 (P<0.01; Figure 2B).

Activation of the complement system and the formation of MAC resulted in an increased membrane permeability and erythrocyte lysis. In this present study, MAC contents in the clot gradually increased with a marked accumulation at day 3

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and stayed at high levels at day 7. Erythrophagocytosis was observed in the hematoma edge at day 3 (Figure 3A), and hemosiderin deposition was identifiable in the hematoma edge at day 7 (Figure 3A). RBCs in the hematoma center started to be phagocytized by macrophages/microglia at day 7 (Figure 3A). The expression of CD 47 in hematoma was high during the first day after ICH and was significantly reduced at day 3 (P<0.01 versus 4 hours and P<0.05 versus day-1; Figure 3B). It remained at low levels at day 7 (Figure 3B).

CD163 and HO-1–positive cells infiltrated into the hematoma from edge to center after ICH (Figure 4A and 4B). A significant increase of CD163 cells was found at day 3. HO-1 protein levels in the hematoma also increased at day 3 (HO-1/GAPDH: 0.65±0.08 at day 3 versus 0.04±0.01 at 4 hours; P<0.01). The morphological characteristics of the CD 163 and HO-1–positive cells in the clot were microglia/macrophage like.

To investigate whether deferoxamine affects the process of hematoma resolution, the ICH piglets were treated with deferoxamine or vehicle for 3 days. We found that hemoglobin contents in hematoma were significantly higher in the deferoxamine-treated group than in the vehicle-treated group (15.9±1.8 versus 11.8±0.8 mg/g; P<0.05; Figure 5A). Deferoxamine also reduced the MAC content in the clot at day 3 (33.0±6.6 versus 56.2±9.2 ng/g in the vehicle-treated group; P<0.05; Figure 5B). These results suggested that the treatment of deferoxamine reduced the process of hemolysis after ICH, which might be because of alleviating MAC formation.

Whether deferoxamine had an effect on CD 47 and erythrophagocytosis in the clot was also examined. The loss of CD47 in the clot was reduced significantly by deferoxamine treatment at day 3 (CD47/GAPDH: 1.32±0.16 in ICH+deferoxamine group versus 0.74±0.07 in ICH+vehicle group; P<0.05; Figure 5C) and day 7 (P<0.01; Figure I in the online-only Data Supplement). Deferoxamine treatment also caused a significant reduction in infiltrating CD163-positive and HO-1–positive cell in the hematoma at day 3 (Figures 6A and 6B) and day 7 (Figures II and III in the online-only Data Supplement). Western blot analysis showed that the protein expression of HO-1 was decreased in the hematoma in the deferoxamine-treated group at day 3 (HO-1/GAPDH: 0.32±0.04 versus 0.98±0.07 in the vehicle-treated group; P<0.05; Figure 6B) and at day 7 (P<0.05; Figure III in the online-only Data Supplement).

**Discussion**

The major findings of this study are as follows: (1) RBCs in the clot become smaller with time; (2) a gradual increase in
clot MAC levels with a progressive decrease in hemoglobin levels; (3) a steady decrease in clot CD47 levels with time which may be linked to significant erythrophagocytosis; (4) increases in the number of CD 163- and HO-1–positive cells infiltrating into the hematoma with time; and (5) deferoxamine attenuated MAC formation and erythrophagocytosis.

The diameter of RBCs within the hematoma decreased with time after ICH. This may be linked to complement-mediated cell lysis. The present study demonstrates that MAC accumulation in the clot with time. RBCs are sensitive to complement-dependent hemolysis with MAC formation. After complement cascade activation, MAC on the cell membrane forms a pore resulting in membrane permeability changes finally leading to RBC morphological alterations and erythrocyte lysis. The cell membrane abnormalities induce a morphological change in RBC from discocyte to spherocyte with a gradual decrease in RBC diameter. It should be noted, however, that other factors may cause a reduction in RBC size. For example, shrinkage occurs in RBCs undergoing eryptosis, a form of suicidal cell death in erythrocytes. Whether eryptosis occurs in the hematoma needs further study.

Phagocytosis of erythrocytes by microglia/macrophages has a role in hematoma clearance after ICH. The current study examined the natural time course of CD47 expression in the hematoma in a swine ICH model and found that CD47 levels in the clot decrease with time. We also showed that the reduction of CD47 levels in the clot is associated with microglia/macrophage infiltration. CD47, as a “don’t eat me” signal, is expressed on RBCs, and has an important role in regulating erythrophagocytosis. CD47 exerts its inhibitory effect on phagocytosis through binding to inhibitory immunoreceptor signal regulatory protein α expressed by microglia/macrophages. Thus, the CD47-signal regulatory protein α constitutes a negative feedback for erythrophagocytosis. The loss of CD47 expression in RBCs may initiate erythrophagocytosis after ICH and lead to hematoma clearance. Our recent study showed that depleting CD47 in RBCs resulted in faster hematoma clearance with microglia/macrophages being less likely to phagocytize RBCs with CD47 than CD47-deficient RBC. However, it should also be noted that perihematomal brain CD47 levels were increased in this...
pig model of ICH.\textsuperscript{13} The precise role of CD47 in brain cells needs to be studied further.

Microglia are brain-resident phagocytic cells that are activated in response to injury stimuli. The transformation of inactive microglia into phagocytic phenotype is associated with alterations in cell surface receptors.\textsuperscript{23} Evidence suggested that CD163 acts as a hemoglobin scavenger receptor on microglia/macrophage with a role in erythrophagocytosis.\textsuperscript{8} With more studies on microglia/macrophage polarization, CD163 is classified into the marker of alternatively activated phenotype (M2 phenotype, phagocytic phenotype).\textsuperscript{23–25} CD163-mediated hemoglobin/hematoma clearance is involved in the induction of HO-1, a rate-limiting enzyme for heme degradation.\textsuperscript{26} Usually HO-1 is considered as an oxidative stress protein in microglia and related to brain injury after ICH.\textsuperscript{11} A recent study, however, indicated that HO-1 is associated with blood clearance by enhancing erythrophagocytosis after stroke.\textsuperscript{9} In the current study, we found that the infiltration of CD163 and HO-1–positive cells into the hematoma increased with time paralleling hematoma resolution. Whether HO-1 is involved in regulating erythrophagocytosis after ICH should be tested further.

Our studies have showed that deferoxamine has a neuroprotective effect on brain injury after ICH, mainly because of iron detoxification.\textsuperscript{10,27} The present study found that deferoxamine treatment reduces MAC formation in the clot. Less MAC accumulation in the clot may result in reduced RBC lysis and less brain iron overload. Deferoxamine also reduced CD47 loss and microglia/macrophage infiltration after ICH, suggesting that it also affects erythrophagocytosis. In combination, these finding may underlie our previous results showing that deferoxamine slows hematoma resolution after ICH in aged rats.\textsuperscript{12} Our future studies will test effects of a combination therapy (iron chelation plus erythrophagocytosis enhancement) on ICH-induced brain injury.

Although this is a novel study focusing on changes in the hematoma using a large animal model of ICH, there are several limitations: (1) hematoma sizes were not measured;
(2) a causal relationship of MAC accumulation and hematoma clearance was not established; and (3) it is still unclear whether hemolysis or phagocytosis is the main process of clot clearance.

In summary, this study presents data on the natural history of hematoma resolution after ICH, which is modified by deferoxamine. This study provides important information on hematoma resolution and informs future studies on how to manipulate that process.

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Disclosures
None.

References
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Supplemental Figures

**Supplemental Figure I:** CD47 protein levels in the hematoma in vehicle- and DFX-treated groups at 7 days after ICH. Values are mean ± SD, n=4, #p<0.01 vs. vehicle treated group.
Supplemental Figure II: CD163 immunoreactivity and numbers of positive cells in the hematoma in vehicle- and DFX-treated groups at 7 days after ICH. Upper scale bar = 50 µm, lower scale bar = 10 µm. Values are mean ± SD, n=4, #p <0.01.
Supplemental Figure III: HO-1 immunoreactivity and protein levels in the hematoma in vehicle- and DFX-treated groups at 7 days after ICH. Upper scale bar = 50 μm, lower scale bar = 10 μm. Values are mean ± SD, n=4, *p <0.05.