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Early Erythrolysis in the Hematoma After Experimental Intracerebral Hemorrhage

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Abstract

Erythrolysis occurs in the clot after intracerebral hemorrhage (ICH) and the release of hemoglobin causes brain injury but it is unclear when such lysis occurs. The present study examined early erythrolysis in rats.

ICH rats had an intra-caudate injection of 100 µl autologous blood and sham rats had a needle insertion. All rats had T2 and T2* MRI scanning and brains were used for histology and CD163 (a hemoglobin scavenger receptor) and DARPP-32 (a neuronal marker) immunohistochemistry. There was marked heterogeneity within the hematoma on T2* MRI, with a hyper- or isointense core and a hypointense periphery. Hematoxylin and eosin staining in the same animals showed significant erythrolysis in the core with the formation of erythrocyte ghosts. The degree of erythrolysis correlated with the severity of perihematomal neuronal loss. Perihematomal CD163 was increased by day 1 after ICH and may be involved in clearing hemoglobin caused by early hemolysis. Furthermore, ICH resulted in more severe erythrolysis, neuronal loss and perihematomal CD163 upregulation in spontaneously hypertensive rats compared to Wistar Kyoto rats.

In conclusions, T2*MRI detectable early erythrolysis occurred in the clot after ICH, and activated CD163. Hypertension is associated with enhanced erythrolysis in the hematoma.

Keywords

Intracerebral hemorrhage; T2* Magnetic resonance imaging; Hematoma; Erythrolysis; CD163; Hypertension

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Conflict of Interest Ge Dang, Yuefan Yang, Gang Wu, Ya Hua, Richard F. Keep and Guohua Xi declare that they have no conflicts of interest.

Compliance with Ethics Requirements: All institutional and national guidelines for the care and use of laboratory animals were followed.

Introduction

Hematoma lysis, hemoglobin release/degradation and brain iron overload cause brain injury after intracerebral hemorrhage (ICH)(1–5). Previous studies have shown that erythrocyte lysis results in brain edema, neuronal death and neurological deficits in animal models(6–8). However, the natural history of erythrolysis in the clot following ICH has not been well examined and the extent to which early erythrolysis occurs in the hematoma not determined.

Magnetic response imaging (MRI) is useful to detect the intracerebral bleeding and has been used in hyperacute ICH (9, 10). Susceptibility-weighted imaging (SWI) and gradient recalled-echo sequence (i.e. T2*-sensitive) MRI show heterogeneity of signal within the hematoma after ICH and this has been attributed to differences in hemoglobin oxygen status (9, 10). However, it is possible that T2* MRI or SWI are detecting areas of erythrolysis within the hematoma and loss of hemoglobin.

If early erythrolysis occurs in the hematoma it may cause perihematomal neuronal loss and also upregulate CD163, a hemoglobin scavenger receptor. CD163 is mainly expressed on macrophages/microglia and it plays a major role in scavenging free hemoglobin released during erythrolysis (11, 12). In ICH patients, the expression of CD163 increased around the hematoma (13) and our recent study found that CD163 positive cells are present and are significantly increased in the hematoma in the first 24 hours in a pig ICH model(14).

Hypertension is the major cause of spontaneous ICH and it is associated with more severe neurological deficits after ICH (15). Our previous study found more perihematomal neuronal death in spontaneously hypertensive rats (SHRs) compared to Wistar Kyoto (WKY) rats after ICH (16). In hypertensive animals and humans, impairments in erythrocyte deformability have been described (17, 18), but it is unclear whether erythrolysis after ICH is affected by hypertension.

Based on the above, the present study used T2* MRI and brain histology to examine early erythrolysis in the hematoma in rats. The role of hypertension in early erythrolysis, neuronal death and CD163 upregulation was also determined.

Methods

Animal preparation and intracerebral infusion

Animal use protocols were approved by the University of Michigan Committee on the Use and Care of Animals. Male Sprague-Dawley rats (weight 275–300g), and SHR (mean arterial blood pressure: 145 ± 8 mmHg) and WKY rats (103 ± 10 mmHg) at 12 weeks of age (Charles River Laboratory, Portage, MI) were used in this study. Blood pressure was measured using a noninvasive tail cuff pressure system. No blood pressure changes were found after ICH.

Rats were anesthetized with pentobarbital (45 mg/kg) intraperitoneally. A feedback-controlled heating pad was used for maintaining core temperature at 37°C. The right femoral artery was catheterized for continuous blood pressure monitoring, blood collection and monitoring blood gases. Rats were positioned in a stereotactic frame and received an

injection of 100µl blood into the right basal ganglia (coordinates: 0.2 mm anterior, 5.5 mm ventral, 3.5 mm lateral to the bregma)(19, 20). Autologous whole blood was injected at a rate of 10 µL/min by a microinfusion pump. Sham controls received only an intracerebral needle insertion. After injection, the needle was removed and the skin incision sutured closed.

Experimental groups

There are two parts of experiments in this study. (1) Male adult Sprague-Dawley rats had either an intracaudate injection of 100µl autologous whole blood (n=6 per time point) or a needle insertion (sham, n=3 per time point) randomly. All animals had T2 and T2* MRI at days 1, 3 and 7, and the brains were used for histology; (2) SHR (n=12) or WKY rats (n=12) had an intracaudate injection of 100 µl autologous whole blood and all animals had T2 and T2* MRI. Hematoma formation was confirmed by MRI. No animals were excluded. The animals were euthanized at day 1, 3 or 7 and the brains were used for histology. GW preformed surgeries and MRI, and GD and YY did all measurements.

Magnetic resonance imaging and T2* lesion measurement

T2 and T2* weighted MRI was performed at day 1 and day 3 after ICH or sham operation(21–23). Rats were anesthetized with 1.5% isoflurane/air mixture throughout MRI in a 7.0-T Varian MR scanner (183-mm horizontal bore; Varian) while maintaining body temperature through circulated heated air. A double-tuned volume radiofrequency coil was used to scan the head. The following parameters were used: TR/TE = 4000/60ms for T2 MRI and 200/5ms for T2* MRI; FOV = 35×35 mm²; matrix = 256×256; slice thickness = 0.5 mm; slice spacing = 0 mm. Images were analyzed using Image J. The T2* lesion was outlined along the border of the hypointense (dark) signal area including the central iso- or hyper-intense signal area, and the total T2* lesion volume was obtained over all slices and multiplying by section thickness. The iso- and hyperintense signal area located in the center of hematoma was outlined on every slice and the iso- and hyperintense signal volume were obtained as the non-hypo-T2* lesion.

Brain histology and hematoxylin and eosin (H&E) staining

Rats were anesthetized and perfused transcardially with 4% paraformaldehyde. Brains were then sectioned on a cryostat 18-µm thick slices). H&E staining was performed following a regular protocol(24) and used to demarcate the hematoma. On that staining, hematoma areas were identified as having intact or lysed erythrocytes. The total hematoma area and the erythrolysis area were measured using image J. The degree of hematoma erythrolysis was calculated as (erythrolysis area/hematoma area) × 100%.

Immunohistochemistry and Immunofluorescence double labeling

Immunohistochemistry and immunofluorescence double labeling were performed as described previously(24–26). The primary antibodies were rabbit anti-CD163 (1:100 dilution, Abcam), rabbit anti-dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa (DARPP-32, 1:200 dilution, Cell Signaling Technology), mouse anti-CD11b(OX42) protein (1:100 dilution; AbD, MCA275R), polyclonal rabbit anti-glial fibrillary acidic protein

(GFAP) IgG (1:400 dilution; Millipore, MCA360), and mouse anti-neuronal-specific nuclear protein (NeuN) IgG (1:500 dilution; Millipore, MAB377). Negative control sections omitted the primary antibody. To analyze neuronal loss, bilateral DARPP-32 positive areas were outlined on three consecutive sections at the blood injection level with a ten-section interval. Neuronal loss was determined for those three sections as: (Contralateral - Ipsilateral) / Contralateral DARPP-32 positive area. For CD163 positive cell counting, 3 images ($\times 40$ magnification) were taken in the perihematoma area. DARPP-32 is a reliable marker of striatal neuronal death (24, 27). All measurements were repeated three times and the mean value was used.

Statistical analyses

All data in this study are presented as means $\pm$ SD. Data was analyzed with Students' *t* test, ANOVA or Spearman's correlation. Differences were considered significant at $p < 0.05$.

Results

Heterogeneity in hematoma appearance on T2*MRI in Sprague-Dawley rats

Both T2 and T2* weighted imaging can detect the hematoma. On T2 weighted imaging, hematomas had a central hyperintense signal surrounded by a hypointense signal at day 1 (Fig. 1 A; upper panels). On T2* weighted imaging, an iso- and hyperintense signal in the center of hematoma was also observed clearly (Fig. 1 A; lower panels). The hypointense signal in the periphery of hematoma was more obvious on T2* weighted imaging than on T2 weighted imaging. At days 3 or 7 after ICH, a similar appearance pattern was observed (Fig. 1 B). We quantified the ratio of iso- and hyper-intense signal volume (non-hypo-T2* lesion volume) to total T2* lesion volume. This was $16 \pm 5\%$ at day 1, $21 \pm 10\%$ at day 3 (Fig. 1 C) and $37 \pm 5\%$ at day 7 after ICH.

Heterogeneity in hematoma appearance on H & E staining in Sprague-Dawley rats

To determine what pathological changes in the hematoma resulted in the non-hypo T2* area, H & E staining was performed. In the periphery of hematoma (hypo-T2* lesion area) most erythrocytes had normal disk-shaped profiles and were intensely stained by H & E (Fig. 2). In contrast, in the center of the hematoma (i.e. non-hypo T2* area), erythrocytes lost their normally disk-shape and most of the erythrocytes were ghosts (small, pale pink, round shape; Fig. 2). Fig 2A shows an example of the border between the two areas. These different erythrocyte profiles were found at both day 1 and day 3 (Fig. 2). We hypothesize that these changes in erythrocyte profile reflect early erythrolysis with the release of intracellular hemoglobin in the center of the hematoma.

Erythrolysis and neuronal loss in the ipsilateral basal ganglia

DARPP-32 is a neuronal marker in the basal ganglia (24). In this study, DARPP-32 staining was used to assess ICH-induced neuronal loss in the ipsilateral basal ganglia. Three sections at different levels of hematoma of each rat were used for H & E staining, and adjacent sections were used for DARPP-32 immunohistochemistry. There was a positive correlation between the degrees of hematoma erythrolysis and neuronal loss (Fig. 3, $r = 0.791$, $n = 18$, $p < 0.01$). This phenomenon was observed both at day 1 and day 3 after ICH.

Early CD163 upregulation in the ipsilateral basal ganglia after ICH

CD163 is a hemoglobin scavenger receptor that is proposed to have a role in clearing hemoglobin after erythrolysis. Immunoreactivity of CD163 was up-regulated significantly in the ipsilateral basal ganglia after ICH in Sprague-Dawley rats by day 1 (Fig. 4). CD163 positive cells in the ipsilateral basal ganglia increased to 303 ± 128 cells/mm² at day 1 and 453 ± 57 cells/mm² at day 3 ($p < 0.01$; Fig. 4). Only a few CD163 positive cells were observed in the ipsilateral basal ganglia of sham controls and in the contralateral basal ganglia of ICH. Double labeling showed that CD163 cells were microglia, neurons or astrocytes (Fig. 5).

Increased non-hypo T2* lesion in the hematomas of SHR compared to WKY rats

Heterogeneity in the T2* signal within the hematoma was also observed in SHR and WKY rats. In SHRs, the ratio of non-hypo T2* lesion volume to total T2* lesion volume was significantly higher than in WKY rats at day 1 (18 ± 5 vs. $8 \pm 5\%$, $p < 0.01$) and day 3 (25 ± 4 vs. $21 \pm 5\%$, $p < 0.05$, Fig. 6). These results suggest that more early erythrolysis occurs in the hematoma in SHR compared to WKY rats.

Greater neuronal loss in SHR than WKY rats after ICH

In Sprague-Dawley rats, neuronal loss correlated with degree of hematoma erythrolysis (Fig. 3). The enhanced erythrolysis in SHR versus WKY rats was also associated with more neuronal loss after ICH as assessed by DARPP-32 staining. At day 1 post-ICH, neuronal loss in SHR was $30 \pm 6\%$, significantly greater than the $14 \pm 4\%$ in WKY rats ($p < 0.05$, Fig. 7). At day 3, neuronal loss was also more severe in SHR than in WKY rats (38 ± 6 vs. $26 \pm 8\%$, $p < 0.05$, Fig. 7).

Greater CD163 upregulation in SHR than WKY rats after ICH

CD163 immunoreactivity was up-regulated in the ipsilateral basal ganglia in both SHR and WKY rats. However, compared to WKY rats, CD163 positive cells were significantly higher in SHRs both at day 1 (290 ± 33 vs. 92 ± 34 cells/mm², $p < 0.05$) and day 3 (531 ± 84 vs. 250 ± 99 cells/mm², $p < 0.05$, Fig. 8).

Discussion

There are several findings in this study: 1) As early as the first day after ICH, the center of hematoma appeared iso- or hyper-intense on T2* weighted MRI while the rim of hematoma was hypo-intense; 2) this corresponded to an area of erythrolysis in the center of hematoma on H&E; 3) the degree of erythrolysis correlated with the severity of ICH-induced neuronal loss; 4) CD163, a hemoglobin scavenger receptor, levels were upregulated early in the ipsilateral basal ganglia after ICH; and 5) the degree of early erythrolysis, ICH-induced neuronal loss and CD163 upregulation in the basal ganglia were greater in SHR than WKY rats.

In the present study, substantial erythrolysis in the hematoma was found by the first day of ICH in the rat models. The main location of early erythrolysis occurred in the center of hematoma. Erythrocyte lysis results in hemoglobin release. Hemoglobin and its degradation products (e.g. iron) then cause neuronal loss around the clot. It is well known that

erythrolysis in the hematoma can cause brain iron overload and delayed brain injury after ICH(1, 6). The present results suggest that erythrolysis can result in not only delayed brain injury but also early brain damage. Our previous studies have showed that erythrolysis may occur very early after ICH. For example, Perl's positive cells were found in the perihematoma area at the first day after ICH(28) and erythrocyte diameters were significantly decreased at the first 24 hours(14). In a rabbit model of ICH, hemoglobin and heme were found in the perihematoma area at 24 hours(29). Other evidence also indicates early erythrolysis after cerebral hemorrhage. Injection of blood into the subarachnoid space in dogs resulted in hemoglobin in CSF by day 1 and a peak on day 2 (30). That the erythrolysis might occur first in the core of a hematoma is supported by recent data on iron distribution within the hematoma after an ICH using X-ray fluorescence microscopy in mice. Those data showed higher iron concentrations on the periphery of the hematoma than in the center (31). Our current study showed a relationship between erythrolysis in the clot and neuronal loss in the perihematoma zone. Future studies should determine the mechanisms of early erythrolysis in the hematoma.

The present data found that early erythrolysis can be detected using T2* MRI. T2* MRI detects both hematoma and iron deposition. Our previous study showed that non-heme iron levels in the perihematoma area were increased with time and reached the peak at day 14 in this rat ICH model(28). It should also be noted that not only erythrolysis can cause the hyper- or isointense signal on T2* MRI. For example, hyper- or isointense signal could be found in the hematoma at the very beginning because clot retraction is not complete and serum accumulates in the clot (Xi, unpublished data). We chose day 1 and day 3 to measure erythrolysis to avoid serum in the clot in the first several hours and delayed significant iron deposition in the perihematoma area. In the acute stages of ICH, areas of hyper- or isointense signal in the hematoma on T2* MRI corresponded with areas of erythrolysis on histology. Marked erythrocyte lysis was found in the core of the hematoma with the formation of erythrocyte ghosts. Such ghosts, which result from erythrolysis and loss of hemoglobin, are the post-hemolytic residues of erythrocytes. In ICH patients, hyper- and isointense signals have also been detected in the center of hematomas by T2* MRI or susceptibility weighted imaging by the first day(9, 10). Previous studies have indicated that the heterogeneous T2* and SWI signals in the hematoma are due to changes in hemoglobin oxygenation status(9, 10). The role of erythrolysis in T2* MRI signal changes in the hematoma should be confirmed in larger ICH animal models and humans. It should also be determined whether or not early erythrolysis within the hematoma occurs in humans.

CD163, a 130 kDa membrane protein, belongs to the "scavenger receptor cysteine rich" (SRCR) superfamily class B and it is expressed in monocytes and macrophages (32). The best known role for CD163 is as a scavenger receptor for hemoglobin released from erythrocytes during intravascular hemolysis (12). CD163 is responsible for clearing hemoglobin both physiologically and pathologically although such clearance may increase greatly during excessive pathological hemolysis (33). While the function of CD163 in clearing hemoglobin in intravascular hemolysis has been well documented, the role of CD163 in ICH is not fully understood. A recent study in patients undergoing intracerebral hematoma evacuation found that CD163 expression increased around the hematoma after ICH (13). Consistent with those results, the present study showed up-regulation of CD163

around hematoma at the acute stage after ICH in rats. CD163 hemoglobin uptake is mainly in the form of a haptoglobin/hemoglobin complex (34). Hemoglobin internalized through interaction with CD163 is transferred to endosomes and subsequently degraded to iron, carbon monoxide and biliverdin by heme oxygenases (35). Therefore, CD163 may have a significant role in hemoglobin clearance around the hematoma. Our present study found that neurons and astrocytes also can express CD163 in the perihematoma area. Recent studies have showed neuronal CD163 (36, 37). The role of CD163 in neurons and astrocytes needs to be determined in future studies.

Early hematoma erythrolysis, as assessed on T2* MRI, was more severe in SHR than in WKY rats. Our previous study found that ICH induces greater neuronal death and neurological deficits in SHR than WKY rats. SHRs also had higher brain ferritin (an iron storage protein) levels and stronger microglia/macrophage activation (16). More early erythrolysis in hematomas may be the cause of the more severe ICH-induced brain injury in SHRs. Hypertension is a prevalent risk factor for ICH, and erythrocyte abnormalities have been widely reported in clinical and experimental hypertension. In hypertensive patients, erythrocyte deformability is significantly reduced (38). The impairment mainly arises from altered erythrocyte membrane properties (17). Hypertensive erythrocytes have an oval shape and display an abnormal membrane skeleton pattern (39). Increased lipid peroxidation and altered tubulin distribution have also been found in erythrocytes of hypertensive patients (40, 41). Moreover, changes in erythrocyte deformability and membrane protein composition have been demonstrated in SHRs (18, 42). In this study, more severe erythrolysis in SHRs might be caused by the abnormal erythrocyte membrane structure and properties.

In conclusion, erythrolysis initiating at the center of hematoma occurs very early after ICH and upregulates perihematoma CD163. The early erythrolysis in the clot can be detected by T2* MRI. Hypertension facilitates the erythrolysis exacerbating ICH-induced neuronal loss. These results suggest that early erythrolysis in the hematoma and CD163 hemoglobin clearance system are potential targets for ICH treatment.

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Figure 1. Representative consecutive T2 and T2* MRIs at day 1 (A) and day 3 (B) after ICH in adult male Sprague-Dawley rats. The ratio of non-hypo-T2* volume to total T2* lesion volume was determined (C). Values are mean $\pm$ SD, n = 6.

Figure 2.

Representative examples of T2 and T2* MRIs and H&E staining at the same level of the hematoma at day 1 (A) and day 3 (B) after ICH in adult male Sprague-Dawley rats. Higher magnification micrographs of the hypo-T2* (1, periphery), the border of hypo-T2* and non-hypo-T2* (2, border), the non-hypo-T2* area (3, center) of the hematoma are also shown. Scale bars are 1 mm for top panels and 10 μ m for lower panels.

A **B** **Figure 3.**

H & E staining and DARPP-32 immunoreactivity in adjacent sections at different levels of the hematoma (A). DARPP-32 staining was used to quantify neuronal loss in the ipsilateral basal ganglia and H&E staining the degree of erythrolysis and the correlation between the two parameters after ICH determined (B).

Figure 4.

Immunoreactivity and quantification of CD163 positive cells in the ipsi- and contralateral caudate at day 1 (A) and day 3 (B) after ICH or a sham operation in adult male Sprague-Dawley rats. Values are mean $\pm$ SD, n=6 in ICH group (per time point) and n=3 in sham-operated group (per time point). Scale bar=50 μ m.

Figure 5. Immunofluorescent double-labelling showed that CD163 positive cells were microglia (OX42 positive), neurons (NeuN positive) and astrocytes (GFAP positive). Scale bar=20 μ m.

Figure 6. Representative consecutive T2* MRIs including the hematoma at day 1 (A) and day 3 (B) after ICH in SHR and WKY rats. The ratio of non-hypo-T2* lesion volume to total T2* lesion volume was quantified (C). Values are mean $\pm$ SD, n=6. *p<0.05, #p<0.01 vs. WKY.

Figure 7. DARPP-32 immunoreactivity at day 1 (A) and day 3 (B) after ICH in SHR and WKY rats. Note the loss of DARPP-32 staining in the ipsilateral (right) caudate putamen. The loss of DARPP-32 staining (neuronal loss) was quantified as: (contralateral - ipsilateral) / contralateral DARPP-32 positive area. Values are mean $\pm$ SD, n=6. Scale bar=1 mm. *p<0.05, #p<0.01 vs. WKY.

Figure 8.

Immunoreactivity and quantification of CD163 positive cells in the ipsilateral caudate putamen at day 1 (A) and day 3 (B) after ICH in SHR and WKY rats. Values are mean $\pm$ SD, n=6. Scale bar=50 μ m. *p<0.05, #p<0.01 vs. WKY.