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Inhibition of fibrin formation reduces neuroinflammation and improves long-term outcome after intracerebral hemorrhage

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ABSTRACT

Background: Intracerebral hemorrhage (ICH) is a severe type of stroke without effective treatment. The coagulation cascade is activated after blood flows into the brain parenchyma. The conversion of fibrinogen to fibrin is an essential step of coagulation processes, but its influences on neuroinflammation and long-term outcome after ICH have not been adequately studied. Hirudin binds to thrombin and inhibits the conversion of fibrinogen to fibrin. We therefore investigated the impact of hirudin treatment on brain inflammation and long-term outcome of ICH in mice.

Methods: Fibrinogen levels were measured in plasma samples from patients with ICH. In mice subjected to collagenase injection, fibrinogen levels were measured in the plasma and brain. The impact of hirudin on neuroinflammation and long-term neurological outcome was determined in ICH mice.

Results: Circulating fibrinogen level was increased in patients with ICH at day 1 and day 4 after onset. In ICH mice, fibrinogen levels in the blood and brain were increased at day 7. Delayed daily administration of hirudin from day 7 to day 28 significantly improved long-term outcome in ICH mice. Hirudin treatment reduced leukocyte accumulation in the brain and shifted microglia toward an anti-inflammatory phenotype. In addition, depletion of microglia in ICH mice diminished the benefit of hirudin in ICH mice.

Conclusions: These results suggest that inhibition of fibrin formation alleviates brain inflammation and improves long-term outcome after ICH.

1. Introduction

Intracerebral hemorrhage (ICH), the most devastating subtype of stroke, accounts for 10–15% of all stroke victims without effective treatment [1,2]. Following the rupture of a cerebral blood vessel, blood accumulates in the tissue around the rupture. The resulting hematoma and the secondary perihematomal edema (PHE) contribute to the high mortality and morbidity after ICH. Evidence has demonstrated that blood components from hematoma can activate the immune system at the site of hemorrhage, leading to concomitant neuroinflammation and progressive brain injury [2–5].

Fibrinogen is a major blood component that enters the central nervous system (CNS) and deposits as insoluble fibrin upon the breakdown of the blood-brain-barrier (BBB). Emerging evidence has demonstrated that fibrin is not merely a maker for BBB damage, but also can augment neuroinflammation by facilitating cytokine secretion from microglia and recruiting leukocytes, thereby leading to exacerbated neural injury [6–10]. In the setting of intracerebral hemorrhage, preclinical and clinical data have revealed that fibrin contribute to edema formation and the level of its precursor (fibrinogen) in the plasma are associated with long-term outcome of ICH patients [10,11]. However, the exact role of fibrin formation on functional outcome and its potential inflammatory mechanism have not been adequately studied in ICH.

Hirudin is a polypeptide originally obtained from the medicinal leech. The C-terminal segment of the hirudin can bind to the fibrinogen recognition site of thrombin and inhibits the conversion of fibrinogen to fibrin during clot formation [12]. Previous evidence has shown the beneficial effects of hirudin to reduce ischemic brain injury [13]. In this study, we therefore investigated the influence of hirudin treatment on brain inflammation and long-term neurological outcome in experimental ICH.

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2. Methods and materials

2.1. Study population

Blood samples were collected from 15 ICH patients that were hospitalized in the Department of Neurology of Tianjin Medical University General Hospital. Of these 15 ICH subjects, 5 were excluded due to their history of autoimmune diseases (rheumatoid arthritis: 2, asthma: 1, autoimmune thyroiditis: 1, other autoimmune diseases: 1) and adoption of immunosuppressant drugs including steroids. 10 ICH patients enrolled did not exhibit complications of infections or tumors at admission (male 5; female 5. Hematoma volume: 21.2 ± 6.7 ml. location of hematoma: basal ganglia). 10 age-matched healthy subjects (male: 5; female: 5) were recruited into this study as controls. Informed consent was obtained from all participants, and the study was approved by the institutional review boards of Tianjin Medical University General Hospital. Blood samples were collected 1 and 4 days after the onset of symptoms. The fibrinogen level was counted by TEG (thromboelastography) in the clinical lab of Tianjin General Hospital.

2.2. Animals, study design and drug administration

All animal experiments were approved by the Committee on the Ethics of Animal Experiments of Tianjin Neurological Institute. All animal experiments were designed, conducted, and reported according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. A total of 103 male C57BL/6 mice (7–8 weeks old) were used in this experiment. The mortality rate was 17.5% (18 of total 103) and exclusion rate was 14.6% (15 of total 103). Mice were excluded based on the clinical score of neurological impairment as measured by modified Neurological Severity Score (mNSS) at 24 h after ICH prior to treatment, as described in behavior assessment section. They were housed in pathogen-free conditions under a standardized light-dark cycle with free access to food and water. All surgeries were operated on animals under anesthesia. ICH mice were randomly divided and assigned to experimental groups. Mice received hirudin (60 ATU/kg) or vehicle by i.p. injection for 21 consecutive days starting at day 7 post ICH. PLX3397 (Selleckchem, Houston, TX) was given by daily oral gavage at 40 mg/kg for 21 days prior to ICH surgery and continued until the end of experiment as previously described [14,15].

2.3. ICH model

ICH was induced by injection of bacterial collagenase in mice, as we previously described [15–18]. Mice were fixed on a stereotactic frame after being anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal (i.p.) injection. A 1-mm-diameter hole was drilled on the right side of the skull (stereotaxic coordinates: 0.5 mm anterior and 2.5 mm lateral to bregma), after which we injected 0.0375 U bacterial collagenase (Type IV-S, Sigma, St. Louis, MO, USA) in 0.5 μl saline restricted to the right caudate nucleus (3.7 mm in depth beneath the surface of the skull) at the rate of 1 μl/min using a 10 μl infusion pump (KD Scientific, Holliston, MA, USA). The needle was gently withdrawn at the rate of 1 mm/min after being held in place for 20 min. Finally, the skull hole was sealed with bone wax and mice were placed in their cages with enough food and water. During surgery, body temperature was maintained at 37 ± 0.5 °C using an electrical blanket. All procedures were conducted to minimize the pain and discomfort of animals.

2.4. Behavioral assessment

The system of behavioral assessment in our study included modified Neurological Severity Score (mNSS) test [19,20], foot fault test [21,22], and cylinder test [23–25]. Two investigators blinded to the mouse groups performed the behavior tests to evaluate the neurodeficits at days 7, 14, 21 and 28 after ICH.

2.4.1. mNss test

The mNSS test, consisting of a set of tests, was used to evaluate many aspects of neurological functions, including motor function and sensory functional reflexes, as previously described [19]. The score was graded from 0 to 18. One point was given if a mouse failed to perform a test. A higher score indicates more severe impairment. The rating scale was as follows: A score of 13–18 indicates severe injury, 7–12 indicates moderate injury, and 1–6 indicates mild injury. Mice with score < 6 or over 13 at 24 h after ICH (prior to treatment) were excluded.

2.4.2. Foot-fault test

Foot-fault test was used to assess a rodent's sensorimotor function [22]. Mice were placed in a grid area (32 cm/20 cm/50 cm (length/width/height)) with a mesh size of 12 mm and allowed to roam freely for 5 min. A foot fault was defined as the limb dropping into the grid hole or resting with the grid at wrist level. Percent foot faults were calculated as the following formula: foot faults / (foot fault + non foot fault steps) × 00.

2.4.3. Cylinder test

Cylinder test was designed to evaluate spatial and motor behavior [24]. Mice were placed in a transparent cylinder 15 cm high and 10 cm in diameter. In the cylinder, mice would rear against the wall and explore the surface of the wall with one or both forelimbs. Mice were recorded for a maximum of 5 min or until the mouse reared 20 times. The percentage of impaired paw usage was assessed using the formula [(contralateral contacts + 1/2 bilateral contacts) / total contacts] × 100.

2.5. Flow cytometry

Flow cytometry was used to quantify the counts of peripheral immune cells and microglia in the brain. Intracellular staining was performed to measure the expression of inflammation-related factors. At day 28 after ICH, mice brain tissues were collected and homogenized through 70 μm nylon cell strainers. After removing myelin using 30% percoll solution, cells were stained with fluorochrome conjugated antibodies as previously described [26–28]. Briefly, all antibodies were purchased from Biolegend (San Diego, CA, USA), unless otherwise indicated. The process of cell staining was performed following the manual instructions. The following antibodies were used: CD45 (30-F11), CD11b (M1/70), CD3 (145-2C11), CD4 (GK1.4), CD8 (53-6.7), NK1.1 (PK136), CD19 (1D3), Ly6G (1A8), F4/80 (6F12), interlukin-6 (MP5-20F3), IL-10 (JES5-16E3), tumor necrosis factor alpha (TNF-α) (MP6-XT22), TGF-β (TW7-20B9), CD86 (GL-1), CD206 (C068C2). IL-1β (NJTEN3) was ordered from eBioscience (SanDiego, CA, USA). Flow cytometry was conducted on FACS Aria flow cytometer and data were analyzed by Flow Jo 7.6.1 software.

2.6. ELISA

The fibrinogen contents of ICH tissues in mice were quantified using an ELISA assay (Abcam, Cambridge, United Kingdom). After plasma collection using EDTA and liver separation, mice were transcardially perfused and ipsilateral striatum regions weighed 100 mg were collected at day 7. Then brain or liver tissue was homogenized in 500 μl chilled 1 × Cell Extraction Buffer PTR, followed by incubation on ice for 20 min and centrifugation at 18000 ×*g* for 20 min at 4 °C. The supernatants were transferred into clean tubes and the sample protein concentration were quantified using a BCA protein assay (Solarbio, Beijing, China). At the same time, each ELISA sample (brain, liver, plasma) or standard was added to the appropriate well with the antibody cocktail, followed by seal and incubation for 1 h at room temperature on a plate shaker set to 400 rpm. After triple washing, 100 μl

TMB Substrate was added to each well and incubated for 10 min in the dark on a plate shaker set to 400 rpm. Finally, 100 μl stop solution was added and the mixture was measured at a wavelength of 450 nm, as previously described [29].

2.7. Statistical analysis

Results were analyzed by investigators blinded to the treatment. GraphPad Prism software was used. Two-tailed unpaired Student's *t*-test was used to determine the significance of differences between two groups. One-way ANOVA followed by a Tukey *post hoc* test was used for 3 or more groups. A two-way ANOVA accompanied by a Bonferroni *post hoc* test was performed for multiple comparisons. Statistical significance was set at $p < 0.05$. Data are shown as mean ± SD.

3. Results

3.1. Increased fibrinogen level in patients with ICH and a mouse model of ICH

Fibrinogen is one key blood-derived molecule that contributes to coagulation and neuroinflammation. To determine the levels of fibrinogen in ICH patients, we obtained blood samples from patients at day 1 and day 4 after ICH onset. A significant increase in plasma fibrinogen levels was seen in ICH patients [control: 2.8 ± 0.3 versus ICH-day 1: 3.2 ± 0.4, (mg/ml), $p = 0.04$]; [control: 2.8 ± 0.3 versus ICH-day 4: 3.5 ± 0.4, (mg/ml), $p = 0.001$] (Fig. 1).

In a mouse model of ICH induced by collagenase injection, we measured the levels of fibrinogen in the plasma, brain and liver homogenates. As shown in Fig. 2, we found a significant increase of fibrinogen in the plasma [control: 99. 9 ± 40.2 versus ICH: 171.9 ± 52.6, μg/ml), $p = 0.02$], and brain tissues [control: 0.03 ± 0.02 versus ICH: 0.1 ± 0.06, μg/mg), $p = 0.001$] at day 7 after ICH. In addition, we found an increased trend in liver tissues [control: 1.8 ± 0.4 versus ICH: 2.2 ± 0.1, μg/mg), $p = 0.08$]. Together, these results suggest an increase of fibrinogen in the circulation after ICH onset.

3.2. Hirudin improves long-term outcome after ICH in mice

To determine the potential role of fibrinogen in ICH pathology, we used a small molecule, hirudin, binds to thrombin to block the conversion of fibrinogen into fibrin [30]. To avoid the potentially increased bleeding caused by fibrinogen inhibition during the early time points after ICH, we initiated hirudin treatment at delayed time points after ICH. Mice received hirudin (60 ATU/kg) or vehicle by i.p. injection for 21 consecutive days starting at day 7 post ICH. ICH mice receiving hirudin treatment displayed an improved performance in neurological assessments including mNSS, foot fault test and cylinder test (Fig. 3).

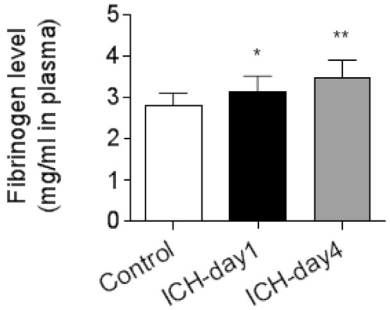

Fig. 1. Plasma fibrinogen levels in patients with ICH.
Blood samples were collected from the patients at the indicated days after ICH onset. Blood samples from healthy individuals were used as controls. Plasma fibrinogen contents were measured by TEG (thromboelastography). Control group: $n = 10$; ICH group: $n = 10$. Data are expressed as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$.

3.3. Hirudin reduces leukocyte accumulation and modulates microglial phenotype

Leukocyte accumulation and microglia activation are pivotal factors involved in inflammation and secondary damage after ICH [31]. To explore the effect of fibrinogen on neuroinflammation, we examined leukocyte and microglia responses in ICH mice receiving hirudin. Gating strategy is shown as in Fig. 4a. At day 28 after ICH onset, no significant alterations of microglia counts were seen in ICH receiving hirudin versus vehicle control [vehicle: 162.5 ± 24 versus hirudin: 150 ± 36, ($\times 10^3$/per brain), $p = 0.5$] (Fig. 4b). However, the counts of leukocytes ($CD45^{high}$), including natural killer (NK) ($CD3^{-}NK1.1^{+}$) [vehicle: 4.3 ± 1.7 versus hirudin: 1.6 ± 0.4, ($\times 10^3$/per brain), $p = 0.003$] and $CD8^{+}$ T cells [vehicle: 11.5 ± 2.6 versus hirudin: 8.3 ± 1.5, ($\times 10^3$/per brain), $p = 0.03$] were significantly reduced in ICH mice receiving hirudin (Fig. 4c).

Upon activation, microglia can be polarized into a cytotoxic (pro-inflammatory) or pro-repair (anti-inflammatory) status depending on the context of disease [32,33]. To investigate the phenotype and functional diversity of microglia after hirudin treatment in ICH, we examined the expression of inflammation-related factors in microglia by flow cytometry (Fig. 4a). In ICH mice receiving hirudin, the number of microglia expressing CD86 [vehicle: 4.0 ± 0.6 versus hirudin: 3.2 ± 0.5, $\times 10^3$/per brain, $p = 0.04$], IL-6 [vehicle: 5.9 ± 0.6 versus hirudin: 4.6 ± 0.5, $\times 10^3$/per brain, $p = 0.003$], IL-1β [vehicle: 5.3 ± 0.9 versus hirudin: 3.8 ± 0.6, $\times 10^3$/per brain, $p = 0.008$] and TNF-α [vehicle: 8.4 ± 1.5 versus hirudin: 6.7 ± 1.0, $\times 10^3$/per brain, $p = 0.04$] were significantly decreased, while the numbers of microglia expressing CD206 [vehicle: 4.6 ± 1.0 versus hirudin: 5.8 ± 0.6, $\times 10^3$/per brain, $p = 0.03$] and IL-10 [vehicle: 1.2 ± 0.3 versus hirudin:1.6 ± 0.4, $\times 10^3$/per brain, $p = 0.04$] were increased (Fig. 4d). Collectively, these results suggest that inhibition of fibrin formation alleviates brain inflammation after ICH.

3.4. The benefit of hirudin in ICH mice involves microglia

Because hirudin treatment shifted microglia toward anti-inflammatory phenotype, we sought to determine whether microglia would contribute to the protective effects of hirudin in ICH. Using a colony-stimulating factor 1 receptor (CSF1R) inhibitor PLX3397, we depleted microglia prior to ICH induction as we previously reported [34]. PLX3397 treatment continued until the end of experiments (Fig. 5a). > 90% of microglia ($CD11b^{+}CD45^{int}$) were depleted in mice receiving PLX3397 for 21 days (Fig. 5b). Importantly, we found that the benefit of hirudin in ICH mice was diminished in ICH mice receiving PLX3397 (Fig. 5c–e). These results indicate that the benefit of hirudin in ICH mice involves microglia.

4. Discussion

This study provides novel evidence that inhibition of the conversion from fibrinogen to fibrin using hirudin reduces neuroinflammation and improves ICH recovery. The benefit of hirudin is associated with reduction of leukocyte accumulation and modulation of microglial response. In addition, the benefit of hirudin treatment was lost in ICH mice subjected to depletion of microglia. Collectively, our results demonstrate that inhibition of fibrin formation might contribute to the long-term recovery after ICH and warrant further related mechanistic studies.

Fibrinogen is a circulating glycoprotein in vertebrates. During tissue and vascular injury, it is converted enzymatically by thrombin to fibrin and subsequently to a fibrin-based blood clot. In ICH, fibrinogen content is related to the pathology and neurological outcome. Reportedly,

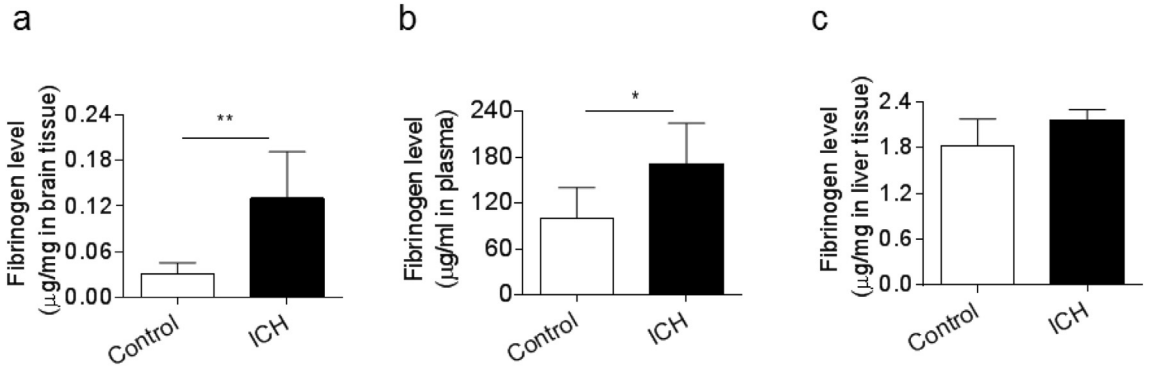

Fig. 2. Fibrinogen levels were increased in the brain and plasma at day 7 after ICH in mice.
ICH was induced in C57BL/6 mice by injection of collagenase. At day 7 after ICH, brain tissue, plasma and liver tissue of ICH mice were harvested for ELISA analysis. The quantification of fibrinogen level in the brain (a), plasma (b) and liver (c). $n = 6$ mice per group. Data are expressed as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$.

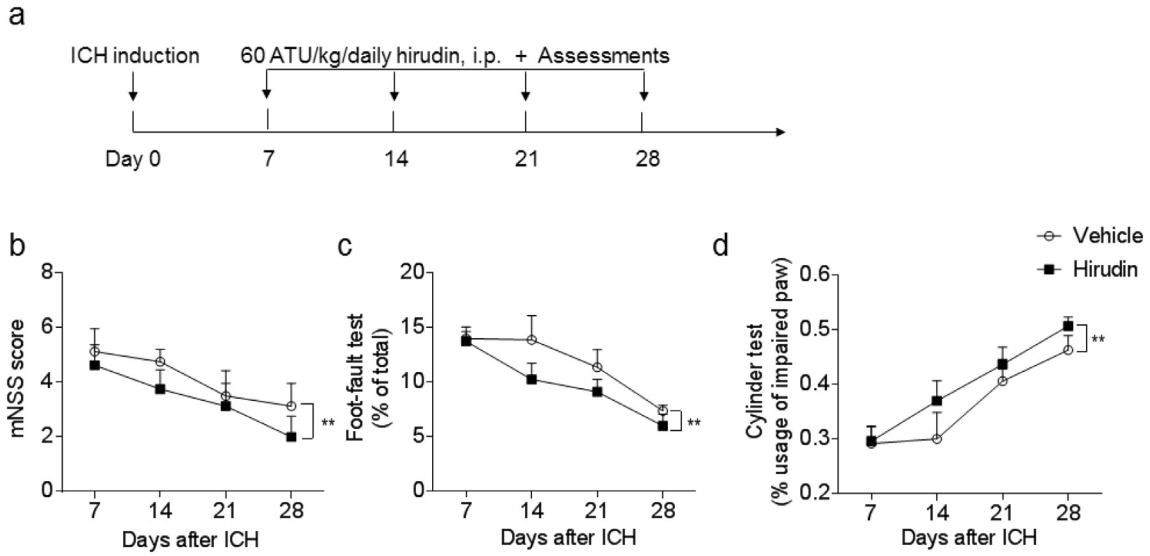

Fig. 3. Hirudin treatment improved long-term neurological outcome after ICH in mice.
ICH was induced in C57BL/6 mice by injection of collagenase. a. Flow chart illustrates hirudin administration and experimental design. C57BL/6 mice were given hirudin (60ATU/kg) or an equal volume of saline by daily intraperitoneal (i.p.) injection, starting from day 7 to day 28 after ICH. At days 7, 14, 21, 28 after ICH, neurological tests were performed to detect the motor, sensory, reflex and balance functions in groups of mice. Neurological function was evaluated by modified Neurological Severity Score (mNSS) (b), foot fault test (c) and cylinder test (d). Data are presented as mean ± SD, $n = 8$ mice per group. $^{**}p < 0.01$.

plasma fibrinogen would drop quickly from the baseline level within minutes after onset of ictus injury. During the following inflammation process, the plasma fibrinogen level would become higher than normal for several days or weeks [35]. Circulating fibrinogen level has been identified as an indicator of worse outcome in hemorrhagic diseases [11]. However, the exact contribution of fibrin formation to the neurological outcome and tissue inflammation is poorly understood in ICH. Our study shows that fibrinogen level in the brain was significantly increased during the late stage of ICH. Delayed blockade of fibrinogen conversion to fibrin augments ICH recovery. These findings support the detrimental role of fibrin formation during the late stage of ICH. Together with results from a previous study [36], our findings provide new evidence that inhibition of fibrin formation is beneficial in ICH.

Previous studies have demonstrated that ICH causes an inflammatory reaction that includes activation of microglia and recruitment of blood-derived leukocytes. Microglia, as brain resident immune cells, are the first responders to brain injury. Evidence indicates that ICH induces microglia activation and polarization. In an autologous blood murine model of ICH, microglial activation occurs as early as 4 h after ICH, and can persist for up to 4 weeks. Genetic profiling of microglia reveals that microglia obtain a phenotype associated with ICH recovery starting at day 7 and persist until day 28 after onset [37–40]. Considering that the conversion of fibrinogen to fibrin is involved in the interactive processes between coagulation and inflammation, we therefore postulate that hirudin may alleviate the brain's inflammatory milieu to confer its protective effect of neurological function after ICH. In support of these view, we found that hirudin treatment reduces leukocyte accumulation and shifts microglia toward anti-inflammatory phenotype. In addition, the benefit of hirudin in ICH mice was reduced after depletion of microglia, suggesting the contribution of microglia to the improved long-term outcome after hirudin treatment. Fibrin formation was reported to activate microglia and induce their expression of antigen-presenting genes [10]. This could be caused by the binding of fibrin to the CD11b/CD18 integrin receptor in microglia or macrophages [10,41]. Together with the reduction of accumulated peripheral leukocytes, these processes could be involved in the reduced neuroinflammation and ICH injury after hirudin treatment.

Owing to the multiple functions of thrombin, it is worthwhile to mention that the beneficial role of hirudin might be related to other mechanisms including inhibition of cytotoxicity to brain cells or reduction of thrombin binding to its receptor (protease-activated receptor-1, PAR-1). The thrombin-PAR1 pathway has been identified as key contributor to microglia activation in neural injury [42]. Future studies are required to test these speculations [36,43,44].

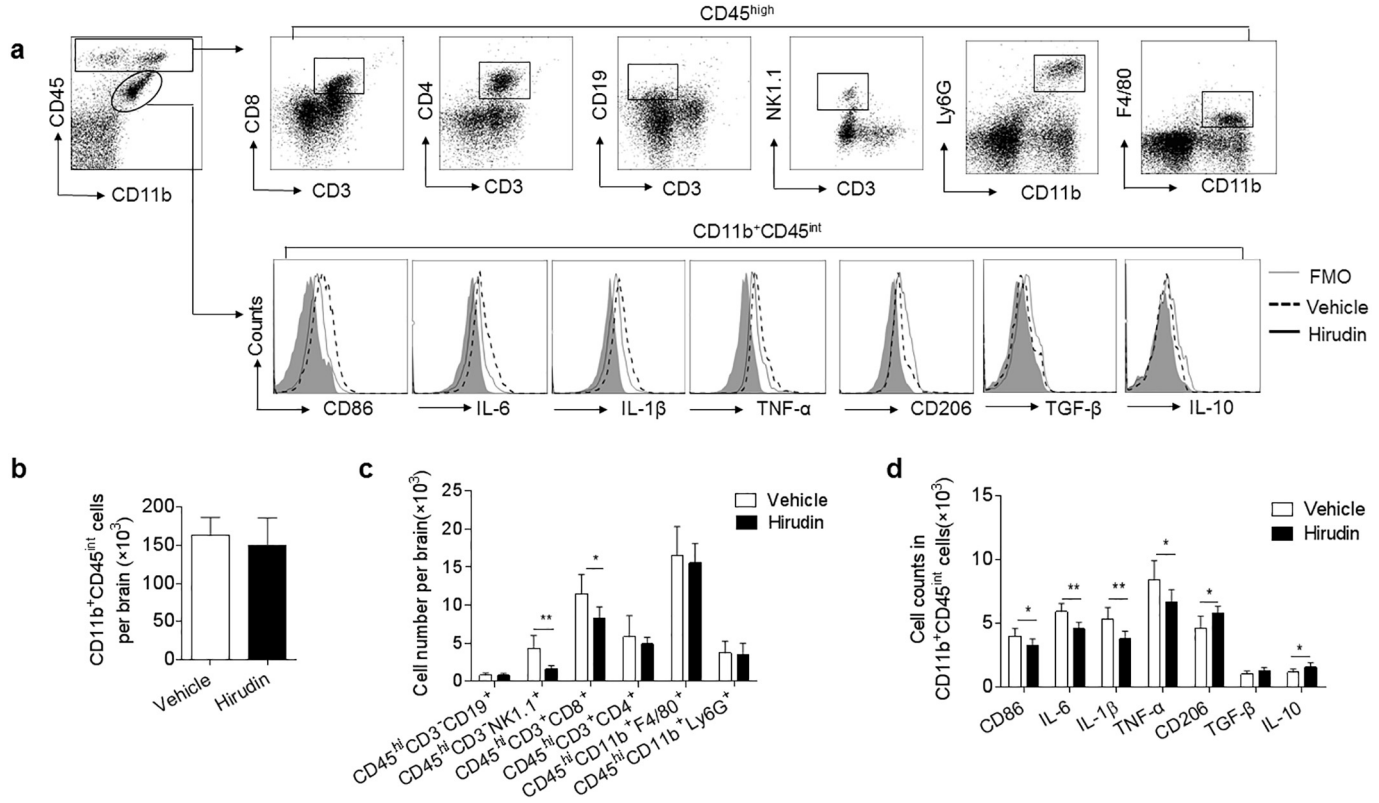

Fig. 4. Hirudin treatment reduced leukocyte accumulation and shifted microglia toward anti-inflammatory phenotype after ICH in mice. ICH was induced in C57BL/6 mice by injection of collagenase. At day 28 after ICH, ICH mice receiving vehicle or hirudin treatment were sacrificed and brain tissues were obtained for flow cytometry analysis. a. Gating strategy of microglia ($CD11b^+CD45^{int}$) and inflammation-related factors (CD86, IL-6, IL-1β, TNF-α, CD206, TGF-β, and IL-10), as well as peripheral immune cells ($CD3^-CD19^+$, $CD3^-NK1.1^+$, $CD3^+CD8^+$, $CD3^+CD4^+$, $CD11b^+F4/80^+$, $CD11b^+Ly6G^+$). b–c. Bar graphs show counts of indicated cell subsets in the ICH brain. d. Counts of microglia expressing CD86, IL-6, IL-1β, TNF-α, CD206, TGF-β, or IL-10 in the ICH brain. n = 6 mice per group. Data are presented as mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$.

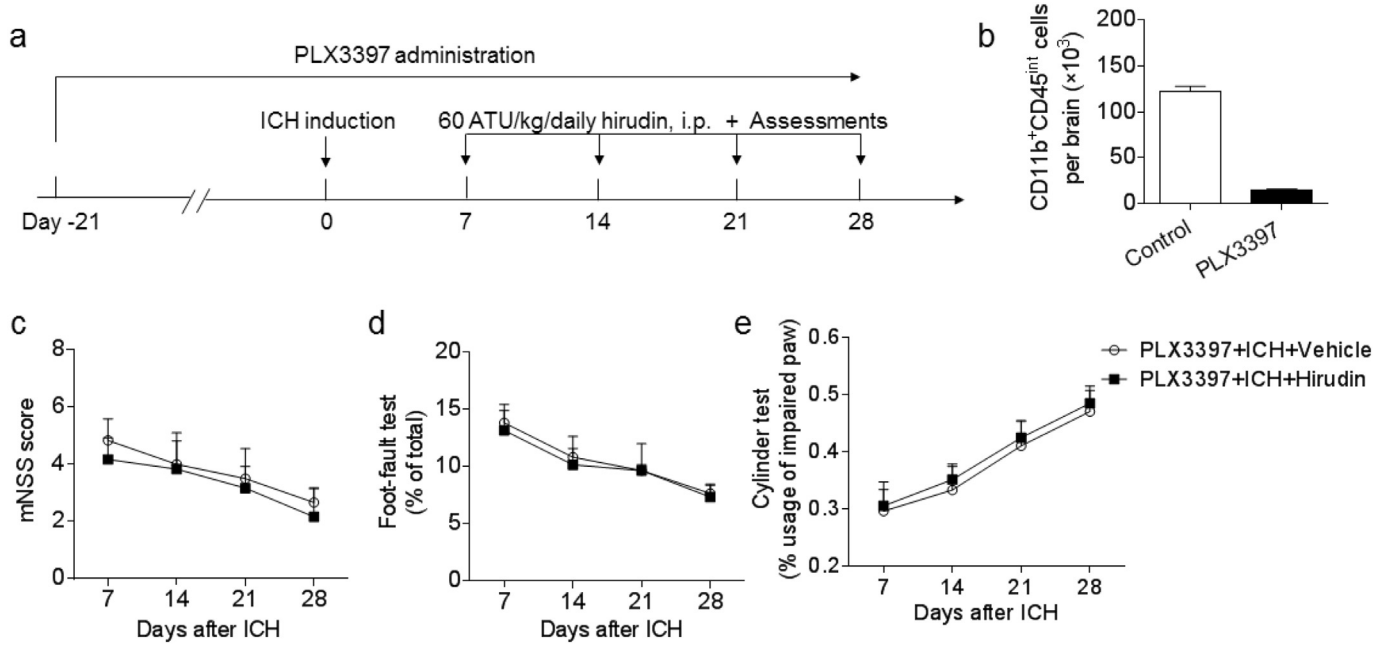

Fig. 5. Depletion of microglia reduced the benefit of hirudin in ICH mice. ICH was induced in C57BL/6 mice by injection of collagenase. a. Flow chart illustrates the experimental design. Mice were given PLX3397 (60 mg/kg) for 21 days prior to ICH induction and the treatment continued until the end of experiment. Thereafter, these mice received daily injection of hirudin or saline, starting from day 7 to day 28 after ICH. Neurological outcomes were measured at the indicated days after ICH. b. Bar graph shows numbers of $CD11b^+$ $CD45^{int}$ cells in groups of C57BL/6 mice receiving vehicle control or PLX3397 for 21 days. c–e. Line chart show results from modified neurological severity score (mNSS), foot fault test and cylinder test. Data are presented as mean ± SD, n = 6 mice per group.

5. Conclusions

In summary, our findings suggest that inhibition of the conversion of fibrinogen to fibrin using hirudin reduces neuroinflammation and promotes ICH recovery.

Author contributions

M.L. formulated the concept and designed the study. X.L. Z.Z. S.G., L.Z., X.C. and S.L. performed the studies. X.L., Z.Z. S.G., and L.Z. analyzed the data and interpreted the results, M.L. and X.L. wrote the paper.

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Conflict of interests

All authors declare no conflict of interest.

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