Recombinant ADAMTS 13 Attenuates Brain Injury After Intracerebral Hemorrhage

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Background and Purpose—Inflammatory responses and blood–brain barrier (BBB) dysfunction play important roles in brain injury after intracerebral hemorrhage (ICH). The metalloprotease ADAMTS 13 (a disintegrin and metalloprotease with thrombospondin type I motif, member 13) was shown to limit inflammatory responses through its proteolytic effects on von Willebrand factor. In the present study, we addressed the role of ADAMTS 13 after experimental ICH.

Methods—ICH was induced in mice by intracerebral infusion of autologous blood. The peri-hematoma inflammatory responses, levels of matrix metalloproteinase-9 and intercellular adhesion molecule-1, pericyte coverage on brain capillaries, and BBB permeability were quantified at 24 hours. Functional outcomes, cerebral edema, and hemorrhagic lesion volume were quantified at day 3.

Results—Treatment with recombinant ADAMTS 13 (rADAMTS 13) reduced the levels of chemokines and cytokines, myeloperoxidase activity, and microglia activation and neutrophil recruitment after ICH. rADAMTS 13 also decreased interleukin-6 expression in brain endothelial cells stimulated by lipopolysaccharide, whereas recombinant von Willebrand factor reversed this effect. The anti-inflammatory effect of rADAMTS 13 was accompanied by reduced expression of intercellular adhesion molecule-1 and less activation of matrix metalloproteinase, enhanced pericyte coverage of brain microvessels, and attenuated BBB disruption. Furthermore, neutrophil depletion protected against BBB damage, and rADAMTS 13 treatment had no further beneficial effect. Finally, treatment of mice with rADAMTS 13 reduced cerebral edema and hemorrhagic lesion volume and improved neurological functions.

Conclusions—Our findings reveal the importance of rADAMTS 13 in regulating pathological inflammation and BBB function and suggest that rADAMTS 13 may provide a new therapeutic strategy for ICH. (Stroke. 2015;46:2647-2653. DOI: 10.1161/STROKEAHA.115.009526.)

Key Words: ADAMTS 13 ■ blood-brain barrier disruption ■ edema and brain injury ■ inflammation ■ intracerebral hemorrhage

Spontaneous intracerebral hemorrhage (ICH) results from rupture of blood vessels in the brain. It represents ≈10% to 20% of all strokes and is a devastating clinical condition with a 30-day mortality rate of 30% to 55%.1 Currently, there is no proven treatment available for improving ICH outcome. Evidence suggest that ICH is associated with activation of local immune cells and release of inflammatory mediators, which result in enhanced disruption of the blood–brain barrier (BBB), causing an increase in peri-hematoma edema formation and consequent neuronal injury.2-4 Cerebral edema is present in most patients with ICH and is an independent predictor of neurological deterioration.1,12 Therefore, strategies aimed at limiting inflammatory response and BBB dysfunction could be potential therapeutic targets for ICH.

von Willebrand factor (VWF) is a multimeric adhesive protein that is released from endothelial Weibel–Palade bodies during injury or inflammation.5 The multimeric size of VWF is modulated by ADAMTS 13 (a disintegrin and metalloprotease with thrombospondin type I motif, member 13), which processed proteolytically VWF into smaller less-reactive forms.6 Accumulating data indicate that ADAMTS 13 and VWF play an opposite role in thrombus formation and...
inflammation. VWF was reported to promote leukocyte rolling, adhesion, and extravasation during cutaneous inflammation and experimental peritonitis.7,8 Recent studies have also shown that ADAMTS 13 deficiency exacerbated inflammatory cell recruitment and ischemic cerebral injury and that treatment with recombinant ADAMTS 13 (rADAMTS 13) inhibited neutrophil extravasation and reduced cerebral infarction.9–11 Recently, we have shown that treatment with rADAMTS 13 reduced tissue-type plasminogen activator–related BBB disruption and hemorrhagic transformation after ischemic stroke.12

In this study, we examined the role of rADAMTS 13 on ICH-induced brain injury. We found that rADAMTS 13 reduced brain edema and neuronal injury by blocking ICH-triggered inflammatory response and consequent BBB impairment.

Materials and Methods

Intracerebral Hemorrhage Model
All animal studies were approved by the Animal Care and Use Committee of School of Basic Medical Sciences, Fudan University. ICH was induced by the double-blood injection method as described previously.11 Three microliters of rADAMTS 13 (100 ng in PBS; R&D systems, Minneapolis, MN) or PBS was injected intraventricularly into mice 1 hour after the start of autologous blood injection. To assess the intravenous efficacy of the drug, rADAMTS 13 (50 μg/kg) was injected intravenously into mice 1 hour after the start of autologous blood injection. In preliminary experiments, rADAMTS 13 at 25, 50, and 100 μg/kg reduced the brain water content, with the maximal effect observed at 50 μg/kg (Figure I in the online-only Data Supplement). For neutrophil depletion, mice received an intraperitoneal administration of 500 μg monoclonal anti-mouse lymphocyte antigen 6 complex locus G antibody (1A8; BioXCell, West Lebanon, NH) 24 hours before ICH. Detailed information is provided in the online-only Data Supplement.

Cell Culture
Mouse brain endothelial cell line (bEnd.3) was used for in vitro studies (online-only Data Supplement).

Measurements of Interleukin-6 and Interleukin-1β Levels
The levels of interleukin (IL)-6 and IL-1β were assayed using enzyme-linked immunosorbent assay (ELISA) kits (R&D systems) according to the manufacturer’s instructions.

BBB Permeability Evaluation
At 21 hours after ICH, mice were intravenously injected with 2% Evans blue dye (4 mL/kg; Sigma-Aldrich). Three hours later, the amount of extravasated Evans blue dye in the hemorrhagic brain hemispheres was evaluated by spectrophotometry (Thermo Scientific, MA) at 620 nm.12

Myeloperoxidase Activity Assay
See details in the online-only Data Supplement.

Real-Time Polymerase Chain Reaction Assay
The polymerase chain reaction was performed with equal amounts of cDNA in an ABI 7300 polymerase chain reaction machine (Applied Biosystems, Foster City). The relative gene expression levels were calculated as the ratio of target cDNA to β-actin (online-only Data Supplement).

Zymography Analysis
Zymography was performed as described.14,15 Human matrix metalloproteinase-9 (MMP-9; Millipore, Billerica, MA) was used as a gelatinase standard. Gels were scanned and analyzed using Quantity One software (Bio-Rad).

Western Blotting
The effects of rADAMTS 13 on intercellular adhesion molecule-1 (ICAM-1) level were evaluated by using Western blots as described.12,16

Immunohistochemistry
Immunohistochemistry was performed as described.16,17 Primary antibodies used were rat anti-CD31 (PECAM-1, BD Pharmingen, San Diego, CA), rat anti-mouse LY-6B.2 (AbD Serotec, Raleigh, NC), goat anti-ionized calcium-binding adapter molecule 1 (Abcam, Cambridge, MA) and fluorescein isothiocyanate–conjugated rat anti-mouse aminopeptidase N (CD13; BD Pharmingen). For quantification of LY6B- and ionized calcium-binding adapter molecule 14,15 Human matrix metalloproteinase-9 (MMP-9; Millipore, Billerica, MA) was used as a gelatinase standard. Gels were scanned and analyzed using Quantity One software (Bio-Rad).

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labeled cells in the peri-hematomal area, 4 fields from each section were captured under 40× objective. The total numbers of positive cells in the traced area were counted and expressed as per mm². Pericyte coverage was determined as a percentage of CD13-positive area covering CD31-positive area in 0.42 mm² regions.

**Behavioral Measurements**

Behavioral functions were measured using neurological score, rotarod test, and corner turn test in a blind fashion (online-only Data Supplement).

**Brain Water Content Measurement**

Three days after ICH, the cerebral hemisphere was cut into 4-mm-thick block around the needle track. Brain tissues were immediately weighted by an analytic balance and heated at 100°C for 24 hours to obtain the dry weight. The water content was calculated using the following formula: (wet weight−dry weight)/wet weight×100%.

**Measurements of Neuronal Degeneration and Hemorrhagic Lesion Volume**

Degenerating neurons were quantified by summing the number of Fluoro-Jade B–positive cells in four 40× fields of the peri-hematoma area with Imaged software. Hemorrhagic lesion volume (defined by the presence of blood or inflammatory cells) was determined by hematoxylin and eosin staining of 6 coronal sections (400 µm apart).

**Statistical Analysis**

Experiments were designed to produce n=5 for Western blot, ELISA, and histological studies and n=7 to 8 for BBB permeability, brain edema, hemorrhagic lesion volume, and behavioral studies (see details in the online-only Data Supplement). Data are represented as mean±standard deviation (SD). Multiple comparisons were performed using 1-way analysis of variance followed by Bonferroni’s multiple comparison test. Differences were analyzed by using the unpaired 2-tailed Student’s t test when 2 groups were compared. Behavior data were compared using Mann–Whitney U test. Significance was accepted with P values <0.05.

**Results**

Recombinant ADAMTS 13 attenuated ICH-associated cerebral inflammation

It is known that inflammation contributes to hemorrhagic brain injury. We analyzed the gene expression profiles of several chemokines in the hemorrhagic brains 24 hours after ICH. The results revealed that ICH strongly elevated the levels of chemokine (C-X-C motif) ligand 1 (CXCL1), CX3CL1, and the chemokine (C-C motif) receptor 1 (CCR1) when compared with the sham-operated group (Figure 1A). Treatment with rADAMTS 13 after ICH resulted in great decrease in these chemokines compared with vehicle-treated mice. Accordingly, ICH-induced upregulation of myeloperoxidase activity and the expression of the proinflammatory cytokines IL-6 and IL-1β were also significantly reduced by rADAMTS 13 treatment (Figure 1B–1D). Immunohistochemical analysis confirmed that both intraventricular and intravenous administration of rADAMTS 13 significantly reduced microglial activation (Figure 2A) and neutrophil accumulation (Figure 2B) in the ipsilateral hemisphere when compared with the vehicle group. In cultured microvascular endothelial cells, upregulation of IL-6 stimulated by lipopolysaccharide was further increased by recombinant von Willebrand factor (rVWF), but this was attenuated by rADAMTS 13 (Figure 3A). Furthermore, rVWF treatment was able to entirely abolish rADAMTS 13–mediated IL-6 downregulation.

**Recombinant ADAMTS 13 Inhibited the Expression of ICAM-1 and MMP-9 and Reduced BBB Impairment**

Using Western blot, we found that rADAMTS 13 treatment profoundly reduced the expression of ICAM-1 when compared with the vehicle group (Figure 4A). In cultured microvascular endothelial cells, rADAMTS 13 blunted the lipopolysaccharide-induced upregulation of ICAM-1, whereas rVWF reversed this effect (Figure 3B and 3C). We also examined the expression of MMP-9 in the hemorrhagic brains of mice treated with vehicle or rADAMTS 13. MMP-9 has been shown to be upregulated in the brain after ICH and contributed to disruption of the BBB. Gelatin zymographic assays revealed that injection of rADAMTS 13 decreased MMP-9 activity compared with the vehicle group at 24 hours after ICH (Figure 4B).

CD13 is a marker of pericyte. We quantified the coverage of pericyte on brain microvessels by CD13 staining and measured the BBB permeability by Evans blue extravasation in mice 24 hours after ICH. We found that ICH resulted in a marked decrease in pericyte coverage in the ipsilateral hemisphere when compared with the sham-operated group (Figure 4C). Intraventricular administration of rADAMTS 13 significantly increased pericyte coverage and reduced BBB disruption caused by ICH (Figure 4C and 4D). Intravenous administration of rADAMTS 13 also caused a significant

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**Figure 2.** Treatment with rADAMTS 13 reduced ICH-induced microglia activation and neutrophil infiltration. A. Representative images of microglia immunostaining and quantification of activated microglia in the peri-hematoma area 24 hours after ICH in mice treated with either intraventricular (ICV) or intravenous (IV) injection of rADAMTS 13. B. Representative images and quantification of neutrophils immunostaining in the peri-hematoma area 24 hours after ICH in mice treated with either intraventricular (ICV) or intravenous (IV) injection of rADAMTS 13. Nuclei were visualized with DAPI. Bar =25 µm. Values are means±SD. n=5 per group, *P<0.05. ADAMTS 13 indicates a disintegrin and metalloprotease with thrombospondin type I motif, member 13; ICH, intracerebral hemorrhage; and rADAMTS 13, recombinant ADAMTS 13.
and Hemorrhagic Lesion Volume and Improved Functional Outcomes After ICH

Mice were treated with rADAMTS 13 or vehicle 1 hour after the start of autologous blood injection, and their brains were examined on day 3. Quantification of brain water content showed that brain edema in the ipsilateral hemisphere was significantly less in rADAMTS 13–treated mice than in vehicle-treated mice (Figure 5A). Mice were similarly protected when injected intravenously with rADAMTS 13 (Figure 5A, right). In the contralateral hemisphere and cerebellum, rADAMTS 13 failed to affect the brain water content. Consistent with this, neuronal death and hemorrhagic lesion volume were significantly reduced in the rADAMTS 13 group compared with the vehicle group (Figure 5B and 5C). Subsequently, we tested functional outcomes using a battery of behavioral tests, including neurological score, rotarod test, and corner turn test in mice treated with rADAMTS 13 or vehicle. These experiments showed that rADAMTS 13–treated mice displayed significantly lower neurological scores and improved rotarod performance on day 3 compared with the vehicle-treated mice (Figure 6A and 6B). Treatment with rADAMTS 13 also reduced preferential turning in the corner turn test compared with treatment with vehicle, although this effect did not reach statistical significance (*P=0.053; Figure 6C).

Using the collagenase-induced ICH model, we demonstrated that both intraventricular and intravenous administration of rADAMTS 13 did not significantly affect hemorrhage volume compared with vehicle treatment (Figure 5D). This suggests that rADAMTS 13 had no effect on collagenase-induced bleeding.

Discussion

ADAMTS 13 has recently been implicated in the pathophysiology of ischemic stroke. In the present study, we investigated the effects of rADAMTS 13 on hemorrhagic brain injury. We found that rADAMTS 13 attenuated ICH-induced cerebral inflammation and BBB breakdown. As a result, brain edema and neuronal injury were reduced, and functional outcomes were improved.

ICH rapidly triggers proinflammatory chemokines and cytokines, upregulation of inflammatory transcriptional pathways, and expression of adhesion molecule, resulting in leukocyte attracted to the injured brain. Leukocyte infiltration can damage vascular endothelium and brain tissue by producing reactive oxygen species, proteases, and proinflammatory mediators. It was shown that inflammatory stimuli induced secretion of VWF, leading to leukocyte adhesion and transmigration, which contributes to further amplification and propagation of inflammation. Here, we found that treatment with the VWF-cleaving protease rADAMTS 13 significantly attenuated brain levels of proinflammatory chemokines and cytokines, concordant with reduced neutrophils infiltration and microglia activation after ICH. This result complement previous finding that reported increased neutrophil extravasation in ADAMTS 13–deficient mice after stroke and myocardial ischemia. The anti-inflammatory effect of rADAMTS 13 on cultured endothelial cells was reversed by rVWF, suggesting that the effect of ADAMTS 13 on inflammation is modulated by VWF.

Inflammation contributes to BBB disruption. ICAM-1 is associated with inflammation-mediated endothelial dysfunction that facilitates BBB leakage after brain injury. MMP-9 has been well known to play important roles in ICH-mediated...
inflammatory responses and the cerebral microvascular damage, and genetic ablation of MMP-9 or pharmacological inhibition of MMPs was protected from ICH.20,32 In our study, we observed that rADAMTS 13 treatment significantly reduced ICAM-1 expression and MMP-9 activation. These lead to significant reduction in pericyte loss and preservation of the BBB integrity. Furthermore, we showed that rADAMTS 13 lost its protective effects on BBB in neutrophil-depleted mice. These findings suggest that the decreased BBB leakage in rADAMTS 13–treated mice may through a neutrophil-mediated process.

Figure 4. Recombinant ADAMTS 13 attenuated ICH-induced BBB damage through a neutrophil-mediated inflammatory processes. 

A, Representative immunoblot and densitometric quantification of ICAM-1 in the brain extracts from mice treated with vehicle or rADAMTS 13 24 hours after ICH. Values are means±SD. n=5 per group. B, Representative MMP-9 gelatin zymography and densitometric quantification of the zymographic gels in the brain extracts from mice treated with vehicle or rADAMTS 13 24 hours after ICH. Values are means±SD. n=5 per group. C, Representative images and quantitative analysis of CD13-positive pericyte coverage on CD31-positive brain capillaries in the brain of sham-operated mice and mice treated with vehicle or rADAMTS 13 24 hours after ICH. Values are means±SD. n=5 per group.

Figure 5. Effects of rADAMTS 13 on brain edema, degenerating neurons, and hemorrhagic lesion volume after ICH.

A, Brain edema in ipsilateral hemisphere (Ipsi), contralateral hemisphere (Contra), and cerebellum (Cereb) 72 hours after ICH in mice treated with either intraventricular (left) or intravenous injection (right) of rADAMTS 13. Values are means±SD. n=8 per group. *P<0.05. B, Representative images of Fluoro-Jade B (FJB) staining and quantification of FJB-positive neurons in brain sections from mice treated with vehicle or rADAMTS 13 72 hours after ICH. Bar=20 μm. Values are means±SD. n=5 per group. *P<0.05. C, Representative hematoxylin and eosin–stained sections and quantification of the hemorrhagic lesion volume in mice treated with vehicle or rADAMTS 13 72 hours after ICH. The black circled lines indicate the areas of hemorrhagic lesion. Bar=100 μm. Values are means±SD. n=8 per group. *P<0.05. D, Hemorrhagic volume measured 24 hours after collagenase-induced ICH by spectrophotometric hemoglobin assay in mice treated with either intraventricular (left) or intravenous injection (right) of rADAMTS 13. Values are means±SD. n=5 per group. ADAMTS 13 indicates a disintegrin and metalloprotease with thrombospondin type I motif, member 13; BBB, blood-brain barrier; Dep, depleted; ICAM-1, intercellular adhesion molecule-1; ICH, intracerebral hemorrhage; LY6G, lymphocyte antigen 6 complex locus G; MMP, matrix metalloproteinase; and rADAMTS 13, recombinant ADAMTS 13.
inflammatory processes. Edema formation that occurs in the peri-hematoma region is likely because of increased BBB damage, which contributes to consequent neuronal loss and delayed brain injury after ICH.33,34 We found that after 3 days of ICH, mice treated with rADAMTS 13 developed significantly less brain edema and smaller hemorrhagic lesion volume as compared with the control group. These data suggest that the protective effect of rADAMTS 13 on hemorrhagic brain injury is most likely caused by the reduction in inflammation-related BBB dysfunction.

No proven treatments exist for ICH. Our study reveals a critical role of rADAMTS 13 in modulating inflammatory responses and BBB permeability after ICH. We also demonstrate that rADAMTS 13 reduced brain edema and minimized neurological deficit in ICH mice. These results suggest that targeting inflammation and BBB damage by rADAMTS 13 may hold a promise for improving ICH outcomes.

Sources of Funding

This work was supported by grants from the National Natural Science Foundation of China (30971014, 81071062, 81271457, and 81471331), the Natural Science Foundation of Shanghai (14ZR1401800), the Innovation Program of Shanghai Municipal Education Commission (12ZZ005), and the National Program of Basic Research sponsored by the Ministry of Science and Technology of China (2011CB503700-G).

References

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*Stroke*. 2015;46:2647-2653; originally published online August 6, 2015;
doi: 10.1161/STROKEAHA.115.009526

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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World Wide Web at:
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Supplemental Methods

Intracerebral Hemorrhage Model and Experimental Groups

All animal studies were approved by the Animal Care and Use Committee of School of Basic Medical Sciences, Fudan University. Male mice from Shanghai SLAC Laboratory Animal Corporation (Shanghai, China), weighing 25 to 30 g, were used in the entire study. Mice were anesthetized with 1-1.5% isoflurane in 30% oxygen and placed on a stereotactic frame (Narishige Scientific Instrument, Tokyo, Japan). Intracerebral hemorrhage (ICH) was induced by the double blood injection method as described previously. Rectal temperature was maintained at 37°C ± 1.0 using a heating pad. A 1 mm-diameter burr hole was drilled on the skull at the following coordinates from the bregma: 0.2 mm anterior and 2.3 mm lateral. Thirty microliters of autologous blood was collected from the tail artery into a capillary tube without heparin and blown into a Hamilton syringe. A 26-gauge needle was inserted into the left striatum 2.8 mm depth below the skull, and 5 μl of blood was injected at 2 μl/minute by a microinfusion pump. The needle was advanced 0.7 mm further, and the remaining 25 μl of blood was injected at 2 μl/minute after 7 minutes. Once the injection was completed, the needle was left in place for 10 minutes and then slowly removed over 5 minutes, and the burr hole was sealed with bone wax. Ten minutes later, a Hamilton syringe was inserted stereotaxically into the left lateral ventricle (coordinates: 0.2 mm posterior to bregma, lateral 1.0 mm to midline, and 2.0 mm ventral to skull surface). Three microliters of rADAMTS 13 (100 ng in PBS; R&D systems, Minneapolis, MN) or PBS was injected intraventricularly 1 hour after the start of autologous blood injection. The injection was performed over 10 minutes. The needle was then removed, the skin was closed, and the animals were allowed to recover. In preliminary experiments, mice (n = 3 per group) were intravenously administered with rADAMTS 13 at three different dosages (25, 50, 100 μg/kg) 1 hour after the start of autologous blood injection. We found that 25, 50 and 100 μg/kg rADAMTS 13 reduced the brain water content from 80.18 ± 0.85% (in the vehicle-treated group) to 79.47 ± 0.63%, 78.36 ± 0.38%, and 78.53 ± 0.78%, respectively (Supplemental Figure 1). Therefore, 50 μg/kg of rADAMTS 13 was used in subsequent experiments. For neutrophil depletion, mice received an intraperitoneal administration of 500 μg monoclonal anti-mouse lymphocyte antigen 6 complex locus G (LY6G) (1A8; BioXCell, West Lebanon, NH) 24 hours before ICH. Previous studies showed that this antibody treatment depleted neutrophils in mice for up to 2-3 days after injection. IgG isotype control was administered in the same way.

To examine whether ADAMTS 13 might affect hemorrhage, the collagenase-induced ICH model was used. In brief, mice were placed on a stereotactic frame and 0.5 μl of saline containing 0.025 U collagenase VII (Sigma-Aldrich) was injected into the left striatum at coordinates 0.2 mm anterior and 2.3 mm lateral to bregma, and 3.5 mm ventral to the skull surface. Ten minutes after removing the needle, a Hamilton syringe was inserted stereotaxically into the left lateral ventricle. Three microliters of rADAMTS 13 (100 ng in PBS) or PBS was injected intraventricularly into mice. In order to assess the intravenous effect of the drug, rADAMTS 13 (50 μg/kg) was injected intravenously into mice 1 hour after the start of collagenase injection. Vehicle groups received the same volume of PBS injection. Hemorrhage volume was assessed by means of a spectrophotometric hemoglobin assay at 24 hours after ICH, as described.

The number of mice used in each study were as follows: for measurements of myeloperoxidase (MPO) activity, interleukin-6 (IL-6) and IL-1β levels: 5 mice were
used in each group; for immunochemistry quantification of activated microglia, neutrophils, pericyte coverage, and degenerating neurons: 5 mice were used in each group; for western blot quantification of matrix metalloproteinase-9 (MMP-9) and intercellular adhesion molecule-1 (ICAM-1): 5 mice were used in each group; for measurements of relative gene expression of chemokine (C-X-C motif) ligand 1 (CXCL1), CX3CL1 and the chemokine (C-C motif) receptor 1 (CCR1): 5 mice were used in sham group, 7 mice were used in other groups; for measurements of BBB permeability: 7 mice were used in control group (intraventricular vehicle-treated group), 8 mice were used in other groups; for measurements of brain edema, hemorrhagic lesion volume, and behavioral functions: 8 mice were used in each groups.

**Cell Culture**

Mouse brain endothelial cell line (bEnd.3) (American Type Culture Collection, Manassas, VA) was used for in vitro studies. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Camarillo, CA) supplemented with 10% fetal calf serum (Invitrogen) and antibiotics at a concentration of 4×10^5 cells/well in a 6-well plate. Cells were cultured without serum for 24 hours before addition of lipopolysaccharide (LPS, 0.1 μg/ml, Sigma-Aldrich, St Louis, MO), rADAMTS 13 (10 ng/ml, R&D systems) and rVWF (10 μg/ml, Haematologic Technologies, Essex Junction, VT) were added 2 hours after addition of LPS. IL-6 and ICAM-1 were detected at 24 hours.

**MPO Activity Assay**

At 24 hours after ICH, ipsilateral brain hemispheres were dissected and homogenized in 50 mM potassium phosphate buffer, centrifuged and suspended in 1% cetyltrimethylammonium bromide (Sigma-Aldrich) in potassium phosphate buffer. The suspensions were sonicated for 30 seconds with three freeze-thaw cycles in liquid nitrogen. After centrifugation, 40 μL of supernatant was incubated with 100 μL tetramethylbenzidine solution (Sigma-Aldrich), and the reaction was stopped with 100 μL 2N HCl. The optical density was measured at 450 nm (Bio-Rad). MPO activity was expressed in equivalent units by comparison with a reference curve generated using purified MPO (Sigma-Aldrich).

**Real-Time PCR Assay**

The PCR was performed with equal amounts of cDNA in an ABI 7300 PCR machine (Applied Biosystems, Foster City). The relative gene expression levels were calculated as the ratio of target cDNA to β-actin. The primers used were: chemokine (C-X-C motif) ligand 1 (CXCL1) forward 5′-ACTGCACCCAAACCGAAGTC-3′, reverse 5′-CAAGGGAGCTTCAGGGT-CAA-3′, CX3CL1 forward 5′-GCACAGGATGCAGGGCTTAC-3′, reverse 5′-TGTCAGCGCCCTCAA-ACT-3′, chemokine (C-C motif) receptor 1 (CCR1) forward 5′-CTGAGGGCCCAACTGTTCAT-3′, reverse 5′-GGCTAGGGCCAGGTGAT-3′, GAPDH forward 5′-AATGTGTCCGTCGTGGATCTGA-3′, reverse 5′-GATGCCCTGTTCACCACCTTCT-3′.

**Behavioral Measurements**

**Neurological score.** Mice were scored for neurological deficit as follows: 0, no neurological deficit; 1, forelimb weakness; 2, spontaneous circling; 3, partial paralysis on one side; 4, absence of spontaneous movement or unconsciousness; 5, death.
**Rotarod test.** Mice were placed on an accelerating rotating rotarod cylinder (Med Associates Inc., St Alban's, VT), and the time the animals remained on the rotarod was recorded. The speed was increased from 4 to 40 rpm within 5 minutes. A trial was terminated if the mouse dropped off the rod or gripped the device and spun around for 2 consecutive revolutions. Mice were trained for 3 days before surgery.

**Corner turn.** Mice were allowed to enter into a corner consisting of two boards attached on one side at an angle of 30 degrees. Ten trials were performed for each animal and the percentage of left turns was calculated. All behavior tests were performed in a blind fashion.
Supplemental Figure I

![Graph showing brain water content (%) for different conditions and treatments.](image-url)
Supplemental Figure I. Brain edema in ipsilateral hemisphere (Ipsi), contralateral hemisphere (Contra) and cerebellum (Cereb) 72 hours after ICH in mice treated with intravenous injection of 25, 50, and 100 μg/kg rADAMTS 13. Values are mean ± SD. n = 3 per group.
References


