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Role of PDGF-D and PDGFR-β in neuroinflammation in experimental ICH mice model☆

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Abstract

Objective—Inflammation plays a key role in the pathophysiological processes after intracerebral hemorrhage (ICH). Post-ICH macrophages infiltrate the brain and release pro-inflammatory

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Conflict of interest

None.

factors (tumor necrosis factor-α), amplifying microglial activation and neutrophil infiltration. Platelet-derived growth factor receptor-β (PDGFR-β) is expressed on macrophages and it's activation induces the recruitment of macrophages. Platelet-derived growth factor-D (PDGF-D) is an agonist with a significantly higher affinity to the PDGFR-β compared to another isoform of the receptor. In this study, we investigated the role of PDGF-D in the pro-inflammatory response after ICH in mice.

Methods—A blood injection model of ICH was used in eight-week old male CD1 mice (weight 30 g). Some mice received an injection of plasmin or PDGF-D. Gleevec, a PDGFR inhibitor, was administered at 1, 3 or 6 h post-ICH. Plasmin was administered with or without PDGF-D siRNAs mixture or scramble siRNA. A plasmin-antagonist, ε-Aminocaproic acid (EACA), was co-administrated with the blood. The effects of ICH and treatment on the brain injury and post-ICH inflammation were investigated.

Results—ICH resulted in the overexpression of PDGF-D, associated with the infiltration of macrophages. PDGFR-inhibition decreased ICH-induced brain injury, attenuating macrophage and neutrophil infiltration, reducing microglial activation and TNF-α production. Administration of recombinant PDGF-D induced TNF-α production, and PDGFR-inhibition attenuated it. A plasmin-antagonist suppressed PDGFR-β activation and microglial activation. Plasmin increased PDGF-D expression, and PDGF-D inhibition reduced neutrophil infiltration.

Conclusion—ICH-induced PDGF-D accumulation contributed to post-ICH inflammation via PDGFR activation and enhanced macrophage infiltration. The inhibition of PDGFR had an anti-inflammatory effect. Plasmin is a possible upstream effector of PDGF-D. The targeting of PDGF-D may provide a novel way to decrease brain injury after ICH.

Keywords

PDGF-D; PDGFR-β; ICH; Neuroinflammation

1. Introduction

Spontaneous intracerebral hemorrhage (ICH) is one of the deadliest stroke subtypes, accounting for about 15% of all strokes (Sutherland and Auer, 2006). Inflammation plays a critical role in the pathophysiology of ICH (Lively and Schlichter, 2012; Manaenko et al., 2010). Macrophages and neutrophils infiltrate the brain and contribute to the post-ICH inflammation by inducing the expression and release of pro-inflammatory cytokines and chemokines, such as tumor necrosis factor-α (TNF-α) (Wang and Dore, 2007; Zhou et al., 2014; Easton, 2013). Platelet-derived growth factor D (PDGF-D) is secreted in its latent form (Fredriksson et al., 2004), and extracellular proteases, such as plasmin, are required for its activation (Fredriksson et al., 2004; Bergsten et al., 2001; LaRochelle et al., 2001).

PDGF-D is the endogenous agonist of the PDGFRs. Although there are indications that PDGF-D has some affinity for the α-isoform of the receptor (Borkham-Kamphorst et al., 2015), most investigators consider PDGF-D as a selective agonist for the PDGFR-β isoform (Fredriksson et al., 2004; Bergsten et al., 2001; LaRochelle et al., 2001; Reigstad et al., 2005; Usuki et al., 1989). Accordingly, PDGF-D administration should primarily activate PDGFR-β. PDGFR-β is largely expressed in macrophages (Heldin and Westermark, 1999;

Inaba et al., 1993) and the activation of the PDGFR-β is involved in macrophage activation (Fuhrman et al., 2009), inflammatory cell infiltration (Nakagawa et al., 2010; Bethel-Brown et al., 2012) and cell migration in the central nervous system (CNS). Gleevec is a new class of anticancer drugs used for treatment of multiply cancers. It is a small molecule tyrosine kinases inhibitor with a high affinity to PDGFRs.

In the present study, we investigated the role of the PDGF-D/PDGFR-β pathway in ICH-induced inflammation and in the development of secondary brain injury after ICH. We hypothesized that PDGF-D, via PDGFR-β activation, induces recruitment and infiltration of macrophages into the brain. We further hypothesized that PDGFR inhibition would attenuate macrophage recruitment and infiltration, consequently reducing TNF-α production, microglial activation, and neutrophil infiltration. This would attenuate the ICH-induced increase in brain water content and neurological deficits. Furthermore, we hypothesized that plasmin is an upstream effector of PDGF-D. To investigate these hypotheses, we first examined the expression of PDGF-D and PDGFR-β after ICH. Next, we inhibited the PDGFR and evaluated the effects of PDGFR inhibition on ICH-induced inflammation, increased brain water content and neurological deficits. Additionally, we administered recombinant PDGF-D into the brains of naïve mice and investigated the ability of PDGF-D to induce inflammation. To verify our hypothesis, that plasmin is an upstream effector of PDGF-D, a plasmin inhibitor (ε-Aminocaproic acid [EACA]) was mixed with autologous blood and co-administered when ICH was induced. Furthermore, plasmin-injected mice were given PDGF-D small interfering RNA to determine, if PDGF-D mediated the effects of plasmin on brain infiltration by neutrophils.

2. Subjects/materials and methods

2.1. Animals

All procedures for this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Loma Linda University. Eight-week old male CD1 mice (weight about 30 g, Charles River, MA, USA) were housed in a 12 hour light/dark cycle at a controlled temperature and humidity, with free access to food and water.

2.2. Intracerebral hemorrhage mouse model

ICH was induced by the autologous arterial blood injection as previously described (Manaenko et al., 2013). Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) (2:1 v/v, intraperitoneal injection). Rectal temperature was maintained at 37.5 °C using a feedback-controlled heating pad. Stereotactic technique was used (Kopf Instruments, Tujunga, CA) to make a scalp incision along the midline and drill a burr hole (1 mm) on the right side of the skull (0.2 mm anterior and 2.0 mm lateral of the bregma). The mouse tail was sterilized with 70% ethanol, and then the tail central artery was penetrated with a sterilized 27G needle. Autologous tail arterial blood was collected in a non-heparinized capillary tube and transferred into a 250 μl Hamilton syringe. The syringe was connected to the microinjection pump, and the needle was inserted into the brain through the burr hole. A total blood volume of 30 μl was injected as follows: the needle was first stopped at 0.7 mm above the target position, and 5 μl of blood was delivered at a rate of 2 μl/min. After 5 min,

the needle was moved to the target coordinates, and the remaining 25 μl of blood was injected at a rate of 2 μl/min. The needle was left in place for an additional 10 min after injection to prevent possible leakage and then slowly withdrawn over 5 min. After the surgery, the skull hole was sealed with bone wax, the incision was closed with sutures, and the mice were allowed to recover. To avoid postsurgical dehydration, 0.5 ml of normal saline was given to each mouse by subcutaneous injection immediately after surgery. Animals were sacrificed for designated experiments 24 and 72 h following ICH surgery.

2.3. Experimental design (Supplemental Fig. 1)

Experiment 1: A PDGFR antagonist, Gleevec (60 mg/kg), was administered intraperitoneally at three different time points (1 h, 3 h or 6 h) post-ICH. Neurological deficits, brain edema were performed at 24 and 72 h.

Experiment 2: Gleevec (60 mg/kg) was administered via intraperitoneal injection 1 h following ICH. Samples for Western blot and immunostaining were collected 24 h after ICH.

Experiment 3: Recombinant PDGF-D was injected into the right basal ganglia in naïve mice (200 ng/2 μl PBS per mouse). Effects of plasmin were evaluated by compartment plasmin-injected animals with plasmin-injected animals treated with Gleevec by Western blot and immune study 24 h post-ICH.

Experiment 4: The plasmin inhibitor EACA (Sigma, 100 μg/2 μl PBS per mouse) was co-injected with blood into the right basal ganglia. Western blot and immunostaining were conducted 24 h after injection.

Experiment 5: The PDGF-D siRNAs mixture or scramble siRNA (100 pmol in 2 μl, OriGene) was administered intraventricularly 24 h before plasmin was injected into the right basal ganglia in naïve mice. Post-assessment included Western blot and immunostaining 24 h after injection.

One hundred and twenty-seven mice were randomly divided into these groups: sham (n = 24), ICH (n = 26), ICH + Gleevec (1 h) (n = 19), ICH + Gleevec (3 h) (n = 14), ICH + Gleevec (6 h) (n = 7), Naïve + PDGF-D (n = 6), Naïve + PDGF-D + Gleevec (n = 6), ICH + EACA (n = 7), Naïve + plasmin (n = 6), Naïve + plasmin + scramble siRNA (n = 6), and Naïve + plasmin + PDGF-D siRNA (n= 6).

The EACA was co-injected with blood into the right basal ganglia. PDGF-D siRNA was delivered 24 h before plasmin injection into the right basal ganglia in naïve mice (Supplemental part, Fig. 1).

2.4. Neurobehavioral test

Neurobehavioral functions were evaluated by an independent researcher blinded to the procedure by the Garcia test and forelimb placement test (Manaenko et al., 2011; Krafft et al., 2014). In the Garcia test, 7 sub-tests (spontaneous activity, axial sensation, vibrissae proprioception, symmetry of limb movement, lateral turning, forelimb walking and climbing) were conducted with a maximum neurological score of 21 (healthy animal). In the forelimb placement test, the animals were held by their trunk, positioned parallel to a table

top and slowly moved up and down, allowing the vibrissae on one side of the head to brush along the table surface. Refractory placements of the impaired (left) forelimb were evaluated, and a score was calculated as the number of successful forelimb placements out of 10 consecutive trials.

2.5. Brain water content measurement

Brain water content was measured as previously described (Krafft et al., 2014). Briefly, mice were decapitated under deep anesthesia. Brains were immediately removed and cut into 4 mm sections around the needle track. Each section was divided into four parts: ipsilateral and contralateral basal ganglia, ipsilateral and contralateral cortex. The cerebellum was collected as an internal control. Each part was weighed on an electronic analytical balance (APX-60, Denver Instrument) and then dried at 100 °C for 24 h to determine the dry weight (DW). Brain water content (%) was calculated as [(WW − DW) / WW] × 100.

2.6. Sample preparation

Mice were euthanized and perfused with 40 ml. of ice-cold PBS. The brains were removed, separated into ipsilateral and contralateral hemisphere, and stored at −80 °C until analysis. Protein extraction was obtained by gently homogenizing brain hemispheres in RIPA lysis buffer (Santa Cruz) with phosphatase inhibitors (Sigma) followed by centrifugation at 14,000*g* at 4 °C for 30 min. The supernatant was collected, and the protein concentration was determined using a detergent compatible assay (Bio-Rad, Dc protein assay). Samples were stored at −80 °C.

2.7. Western blotting

Thirty (30) micrograms of protein was loaded on SDS-PAGE gel. After being electrophoresed, proteins were transferred to a nitrocellulose membrane. The membrane was blocked and incubated with the primary antibody overnight at 4 °C. The primary antibodies were: anti-p-PDGFR-β (1:1000, Santa Cruz), anti-PDGF-D (1:1000, Santa Cruz), anti-MPO (1:1000, Santa Cruz), anti-TNF-α (1:1000, Santa Cruz). The nitrocellulose membranes were incubated with secondary antibodies (1:8000, Santa Cruz) for 1 h at room temperature. Immunoblots were then probed with an ECL Plus chemiluminescence reagent kit (Amersham Biosciences, Arlington Heights, IL) and visualized with the image system (Bio-Rad, Versa Doc, model 4000). All data were analyzed using Image J software.

2.8. Immunofluorescence

Twenty-four hours after ICH, mice were perfused under deep anesthesia with 100 ml of ice-cold PBS followed by perfusion with 30 ml formalin (10%). The brains were removed and fixed in formalin at 4 °C for a minimum of 3 days. Samples were then dehydrated with 30% sucrose in PBS and sectioned with cryostat (CM3050S; Leica Microsystems) in 10 μm coronal slices. Anti-PDGFR-β antibody (1:100, Santa Cruz), anti-PDGF-D (1:100, Santa Cruz), anti-MPO (1:100, Santa Cruz), anti-Macrophages/Monocytes (1:100, Millipore), anti-Iba1 antibody (1:100, Abcam), anti-NeuN (1:100, Abcam), anti-GFAP (1:100, Abcam) were incubated separately or double staining overnight at 4 °C. It was then incubated with the appropriate fluorescence conjugated secondary antibodies (1:200, Jackson Immunoresearch,

West Grove, PA). The slices were visualized underneath a fluorescence microscope (Olympus BX51, Olympus Optical Co. Ltd., Japan), and pictures were taken with MagnaFire SP 2.1B software (Olympus, Melville, NY).

Macrophages and microglia were stained with ED1 and Iba-1 stains and these two types of cells were distinguished by their morphology as previously described (Power et al., 2003). Macrophage positive cells were quantified in the perihematoma region at 24 h using 12 fields per slide.

2.9. Statistics

Data were expressed as mean ± standard error of the mean and analyzed using GraphPad Prism software. Statistical differences between the two groups were analyzed using Student's unpaired, two-tailed *t*-test. Multiple comparisons were statistically analyzed with one-way analysis of variance (ANOVA) followed by Tukey multiple comparison post hoc analysis or Student-Newman-Keuls test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. PDGF-D level was increased after ICH and PDGFR-β expressed on infiltrated blood derived macrophages

Twenty-four hours after ICH, more accumulation of PDGF-D was observed in the ipsilateral (ips) compared to the contralateral hemispheres (contra) ($p < 0.05$) of ICH animals and sham operated animals. No difference between PDGF-D levels in the contralateral hemisphere of ICH animals compared to sham operated animals was detected at this time point (Fig. 1). Immunofluorescence staining revealed that the PDGF-D was expressed on neurons and astrocytes. PDGFR-β was also detected in macrophages (Fig. 1).

3.2. Gleevec improved neurological functions and reduced brain edema at both 24 and 72 h following ICH

At 24 h, all ICH animals demonstrated significant neurological deficits when compared to sham operated animals. (Fig. 2A and B, $p < 0.05$ for Garcia and forelimb placement tests, respectively). Although Gleevec administrated 1 and 3 h post-ICH significantly attenuated ICH-induced neurological deficits, the later treatment with Gleevec (6 h post-ICH) was ineffective (Fig. 2A, B).

At the delayed stage (72 h post-ICH), Gleevec treatment that was started 3 h after ICH significantly improved neurobehavioral function (Fig. 2A and B) and decreased ICH-induced brain edema (ipsi-BG: Gleevec (3 h post-ICH), 81.54 ± 0.21 vs untreated 82.53 ± 0.33, $p < 0.05$; Fig. 2D).

In agreement with the neurological evaluation deficits, we observed brain water content elevation in the ipsilateral basal ganglia of ICH animals compared to sham-operated animals was evaluated at 24 and 72 h post-ICH (Fig. 2C and D: ipsi-BG: 24 h post-operation: ICH, 81.73 ± 0.39 vs sham, 79.26 ± 0.35, $p < 0.05$; 72 h post-operation: ICH, 82.53 ± 0.33 vs sham, 79.53 ± 0.40, $p < 0.05$). The effects of Gleevec treatment on ICH induced brain edema were investigated at 24 and 72 h after ICH. Similar to the 24 h after-ICH treatments, Gleevec

treatment that was started 1 or 3 h post-ICH significantly decreased brain edema in the ipsilateral basal ganglia (ipsi-BG) of treated animals compared to non-treated animals (ipsi-BG: Gleevec (1 h), 80.71 ± 0.16 vs untreated, 81.73 ± 0.39, $p < 0.05$; Gleevec (3 h), 80.81 ± 0.18 vs untreated, 81.73 ± 0.39, $p < 0.05$; Fig. 2). Again, the treatment that was started 6 h after ICH induction was ineffective.

Additionally, 24 h post-ICH, we observed spreading of brain edema in the ipsilateral cortex (ipsi-CX). Brain water content was significantly increased in the ICH compared to the sham animals (ipsi-CX; bICH, 79.95 ± 0.18 vs sham, 79.17 ± 0.18, $p < 0.05$, Fig. 2C). Gleevec treatment showed a non-significant trend towards decreased brain edema.

3.3. Gleevec reduced macrophage number and neutrophil infiltration as well as microglia activation resulting in attenuated TNF-α expression

ICH caused a significant brain infiltration by macrophages at 24 h after ICH. Gleevec treatment started 1 h after ICH significantly reduced this effect ($p < 0.05$, Fig. 3A). Similarly, ICH resulted in an increase in the number of infiltrated MPO positive neutrophils and activated microglia (Fig. 3B and C). Gleevec administrated 1 h post-ICH significantly decreased ICH-induced brain infiltration by neutrophils and microglia activation evaluated 24 h after ICH ($p < 0.05$, Fig. 3B and C).

Compared to sham operated animals, ICH significantly increased phosphorylation of PDGFR-β, TNF-α and MPO expression ($p < 0.05$, Fig. 4 A, A1, A2 and A3) evaluated at 24 h post-ICH. Gleevec administrated 1 h after ICH was able to attenuate the phosphorylation of PDGFR-β, TNF-α and MPO level 24 h post-ICH ($p < 0.05$, Fig. 4 A, A1, A2 and A3).

3.4. PDGF-D promoted TNF-α expression in naïve mice whereas Gleevec attenuated PDGF-D induced TNF-α production

PDGF-D injection into the right basal ganglia increased the level of phosphorylation of PDGFR-β and TNF-α in the ipsilateral hemisphere of injected compared to non-injected animals ($p < 0.05$, Fig. 4 B, B1 and B2). Gleevec administrated 1 h after PDGF-D injection significantly decreased PDGF-D induced elevations of phosphorylation of PDGFR-β and TNF-α at 24 h post-ICH ($p < 0.05$, Fig. 4 B, B1 and B2).

3.5. Plasmin inhibition suppressed both ICH induced PDGF-D expression and PDGFR-β activation as well as microglia activation 24 h post-ICH

The plasmin inhibitor 6-minocaproic Acid (EACA) attenuated ICH-induced accumulation of PDGF-D and phosphorylation of PDGFR-β ($p < 0.05$, Fig. 5A). Additionally, EACA attenuated microglia activation, as demonstrated by a reduction in the number of Iba-1 positive cells (Fig. 5B).

3.6. Plasmin increased PDGF-D and MPO expression in naïve animals, and PDGF-D siRNA attenuated plasmin induced MPO increases

Compared to sham animals, PDGF-D levels were significantly increased in the brains of animals after plasmin injection 24 h post-ICH ($p < 0.05$, Fig. 5C). MPO levels were also increased 24 h after plasmin injection ($p < 0.05$, Fig. 5D). The administration of PDGF-D

siRNA attenuated this effect, and levels of MPO decreased compared to the plasmin group ($p < 0.05$, Fig. 5D).

4. Discussion

ICH is a fatal stroke subtype, and inflammation plays a significant role in the development of secondary brain injury (Wang and Dore, 2007; Zhou et al., 2014). In the present study, we investigated whether 1) ICH induced accumulation of PDGF-D, a recently discovered form of PDGF, 2) PDGF-D accumulation is accompanied bymacrophage recruitment and infiltration into the brain, 3) PDGFR inhibition will attenuate macrophage infiltration, resulting in a decrease of TNF-α production, microglia activation and macrophage infiltration, as well as in reduction of neurological deficits and brain edema after ICH. We also tested whether 4) plasmin is an upstream effector of PDGF-D.

Accumulating evidence suggests that macrophages infiltrate the brain shortly after ICH. Infiltrated macrophages express pro-inflammatory cytokines and chemokines and are able to amplify microglia activation, neutrophil infiltration and, therefore, significantly contribute to the development of brain injury (Wang and Dore, 2007; Zhou et al., 2014; Power et al., 2003; Hammond et al., 2014). One of the isoforms of PDGF receptor, PDGFR-β is highly expressed on macrophages (Heldin and Westermark, 1999) and is involved in the macrophage activation and migration (Fuhrman et al., 2009; Uutela et al., 2004; Wang et al., 2010a,b). PDGF-D is an endogenic agonist with high affinity to PDGFR-β (Fredriksson et al., 2004; Bergsten et al., 2001; LaRochelle et al., 2001; Reigstad et al., 2005; Usuki et al., 1989). PDGF-D is secreted in its latent form and requires extracellular proteases, such as plasmin, for activation (Fredriksson et al., 2004; Bergsten et al., 2001; LaRochelle et al., 2001; Reigstad et al., 2005). PDGF-D was found to induce macrophage recruitment and infiltration through PDGFR-β (Uutela et al., 2004). There is an indication that PDGF-D/PDGFRβ pathway might be involved in the inflammation induced by ICH (Bai et al., 2012).

In the present study, we investigated whether ICH would induce PDGF-D accumulation, and whether the accumulation would be accompanied by increased inflammation and deterioration of brain injury after ICH. We demonstrated that ICH increased the expression of PDGF-D in the ipsilateral hemisphere as early as 24 h after ICH, and that PDGF-D was expressed in both neurons and astrocytes. We also found the PDGFR-β was also expressed on the infiltrated macrophages. Moreover, we demonstrated that PDGF-D administration into the brains of naïve animals significantly increased TNF-α levels. These results confirmed our hypothesis that PDGF-D/PDGFRβ modulates brain infiltration by systemic macrophages and is involved in the inflammatory post-ICH response.

In the next part of our study, we investigated whether PDGFR inhibition would attenuate ICH induced inflammation and subsequently ameliorate development of secondary brain injury after ICH. We used the PDGFR antagonist, Gleevec, to investigate whether PDGFR inhibition would result in an anti-inflammatory effect after ICH and attenuate ICH induced brain injury. We demonstrated that Gleevec treatment significantly attenuated the brain infiltration of both macrophages and neutrophils and resulted in the decrease of the TNF-α levels and microglial activation. We also tested whether Gleevec treatment would reduce

ICH induced brain edema and neurological deficits. Gleevec administered 1 h after ICH preserved the blood brain barrier after ICH, resulting in decreased brain edema and improved neurological function (Ma et al., 2011). In clinical practice, however, some patients cannot be treated within the first hours after disease onset. Accordingly, we expanded the therapeutic window of Gleevec treatment, and administered Gleevec (60 mg/kg) both at 1 h and at two later time points, 3 h and 6 h, after ICH. In agreement with our previous study, Gleevec treatment started 1 h after ICH significantly decreased ICH induced brain edema and improved neurological function. We observed these improvements both 24 and 72 h after ICH. Additionally, treatment started 3 h after ICH attenuated both brain edema and ICH-induced neurological deficits as well. However, Gleevec administrated 6 h after ICH had no effect on the investigated parameters.

Finally, we examined the potential upstream orchestrator of PDGF-D/PDGFR-β activation after ICH. PDGF-D is secreted as a latent form that requires activation by extracellular proteases. Upon activation, PDGF-D binds mostly to PDGFR-β and activates it (Fredriksson et al., 2004; Bergsten et al., 2001; LaRochelle et al., 2001; Andrae et al., 2008). A possible activator of PDGF-D is plasmin. After ICH, coagulation is initiated by the activation of thrombin, followed by fibrin formation. Plasmin cleaves the fibrin, resulting in both clot lysis and generation of fibrin fragments, as well as modulation of the inflammatory response by increasing leukocyte migration, which in turn increases pro-inflammatory cytokine production (Andrae et al., 2008; Niego and Medcalf, 2014). Plasmin plays a significant role in neuroinflammation and blood brain barrier disruption (Zhou et al., 2014; Niego and Medcalf, 2014). Therefore, in the present study, we investigated whether plasmin can activate PDGF-D Plasmin injection into the brain increased PDGF-D production and MPO expression. Inhibition of PDGF-D with PDGF-D siRNA reduced plasmin induced MPO overproduction 24 h after operation in naïve mice. Additionally, the plasmin inhibitor EACA, co-injected with autologous arterial blood into the right basal ganglia of mice, attenuated PDGF-D production and PDGFR-β activation that resulted in the attenuation microglia activation. These findings revealed that plasmin is an upstream regulator of PDGF-D/PDGFR-β system.

It is worth to mention that the PDGFR-β is also highly expressed in pericytes and that the Gleevec treatment may affect pericyte biology as well. Since the pericyte biology was not a goal of the present study, we have not investigated these effects yet. However, we will examine the importance of the pericyte PDGFR-β inhibition in our future studies. In conclusion, our findings indicated that PDGF-D/PDGFR-β pathway contributes to inflammation after ICH by enhancing macrophage infiltration. PDGFR inhibition is protective and has anti-inflammatory effects. Plasmin is an upstream effector of PDGF-D, and therapeutic interventions targeting PDGF-D may provide a novel way to decrease inflammation after ICH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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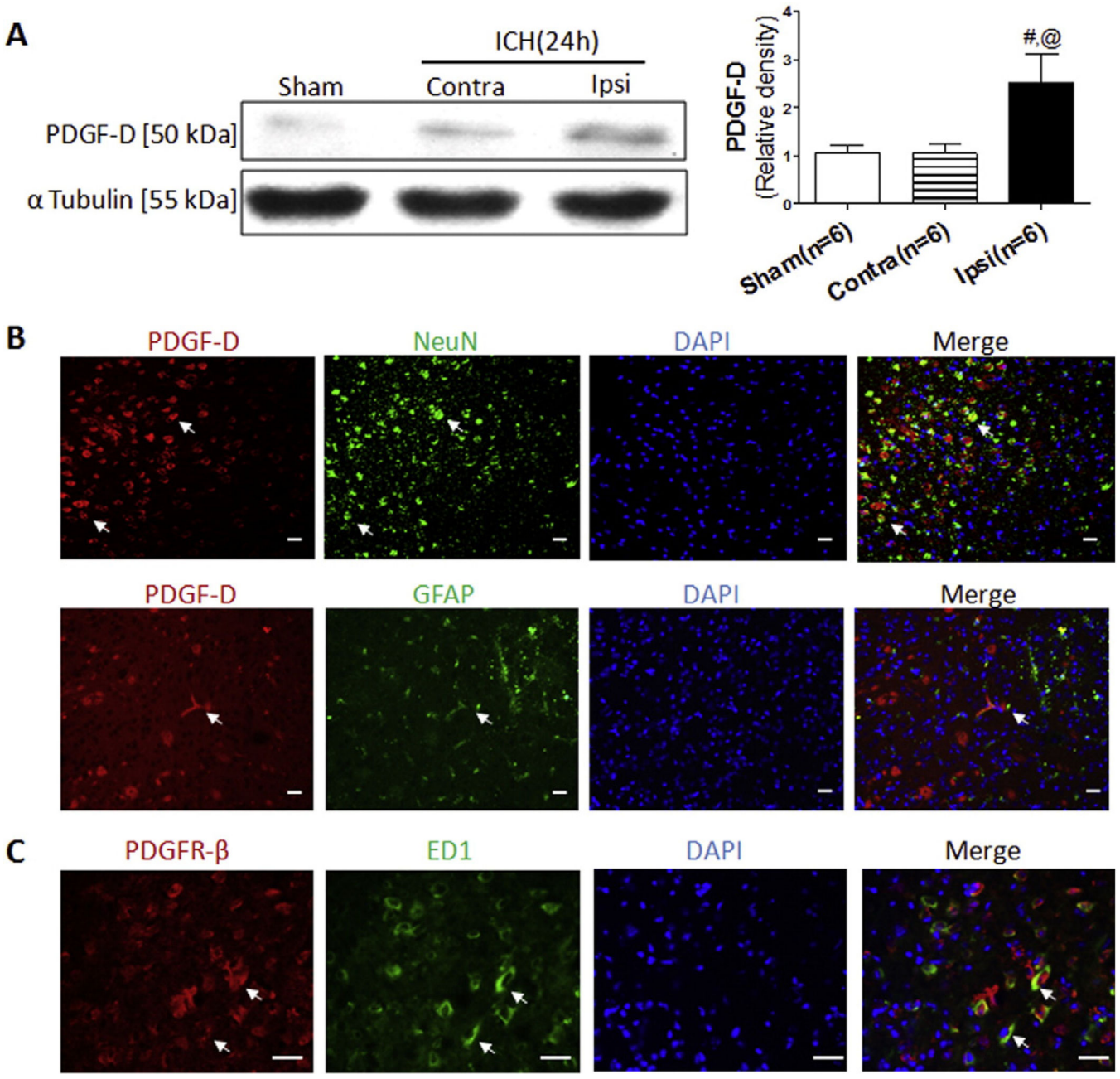

Fig. 1.

ICH increased PDGF-D expression and macrophages infiltration (24 h after ICH). (A1) At 24 h after ICH significant increased expression of PDGF-D expression was observed in ipsilateral hemisphere (ipsi) compared to contralateral hemisphere (Contra) and sham operated animals (n = 6 mice per group. Error bars represent mean ± standard error of the mean. $^{\#}p < 0.05$ vs sham; $^{@}p < 0.05$ vs Contra). (B) Representative photographs of immunofluorescence staining for PDGFD (red) expression in neurons (NeuN, green) and astrocytes (GFAP, green) the perihematomal area 24 h after ICH. (C) Representative photographs of immunofluorescence staining for PDGFR-β (red) expression in infiltrated macrophages (macrophages, green) in the perihematomal area 24 h after ICH. Bar = 50 μm.

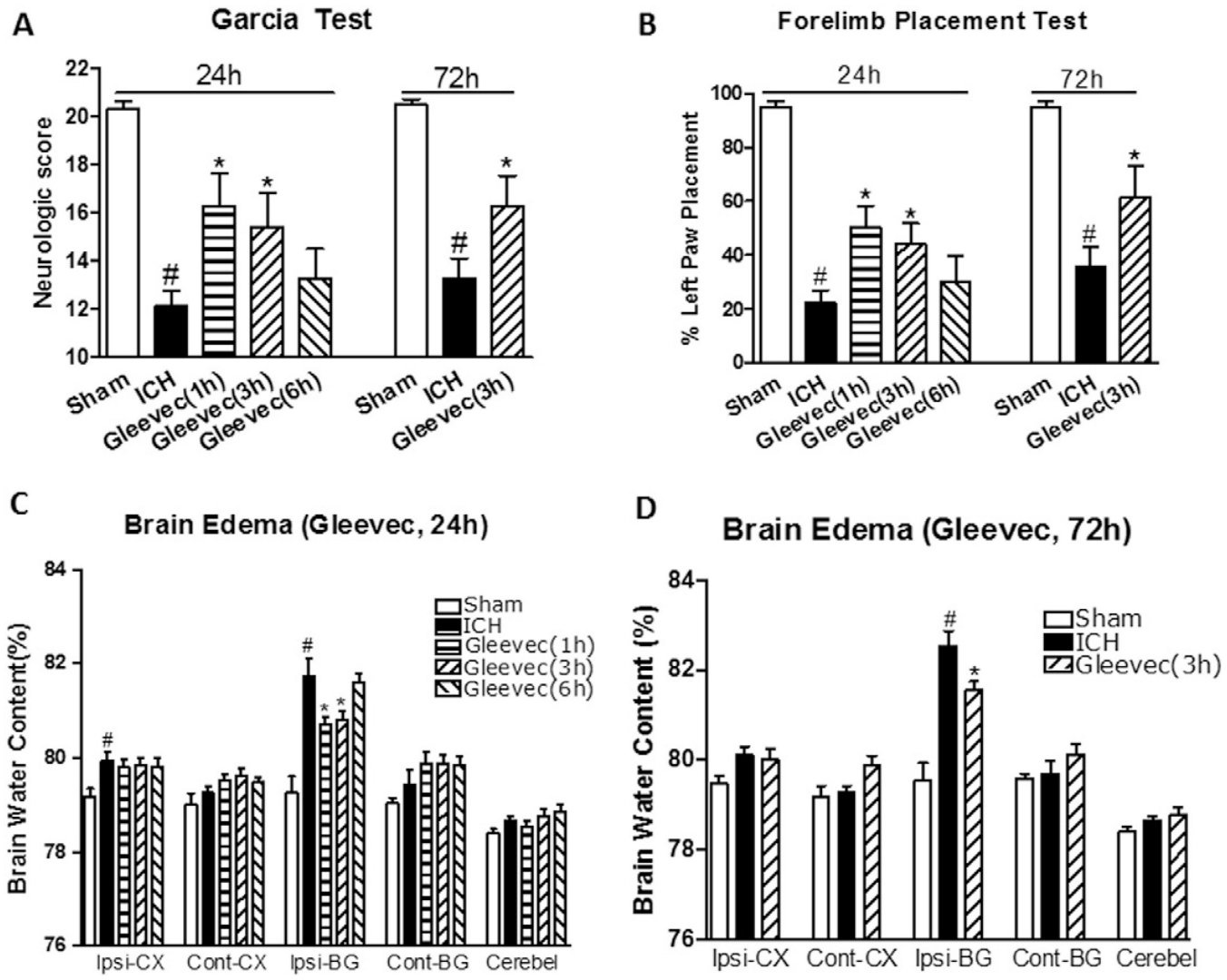

Fig. 2.

Gleevec improved neurological function and reduced ICH induced brain edema at 24 and 72 h following ICH. Gleevec was administered at three time points (1, 3, 6 h) after ICH. Treatment started at 1 and 3 h after ICH significantly improved neurological function on the (A) Garcia test and on the (B) forelimb placement test 24 after ICH. Furthermore, treatments started 3 h after ICH improved neurological function evaluated 72 h after as well (A and B). ICH caused significant elevation of brain water content evaluated at 24 and 72 h after ICH (C and D). Gleevec treatment started at 1 and 3, but not at 6 h, after ICH attenuated ICH induced increase of brain water content (C and D). n = 6 to 7 mice per group. Error bars represent mean ± standard error of the mean. $^{\#}p < 0.05$ vs sham; $^{*}p < 0.05$ vs ICH.

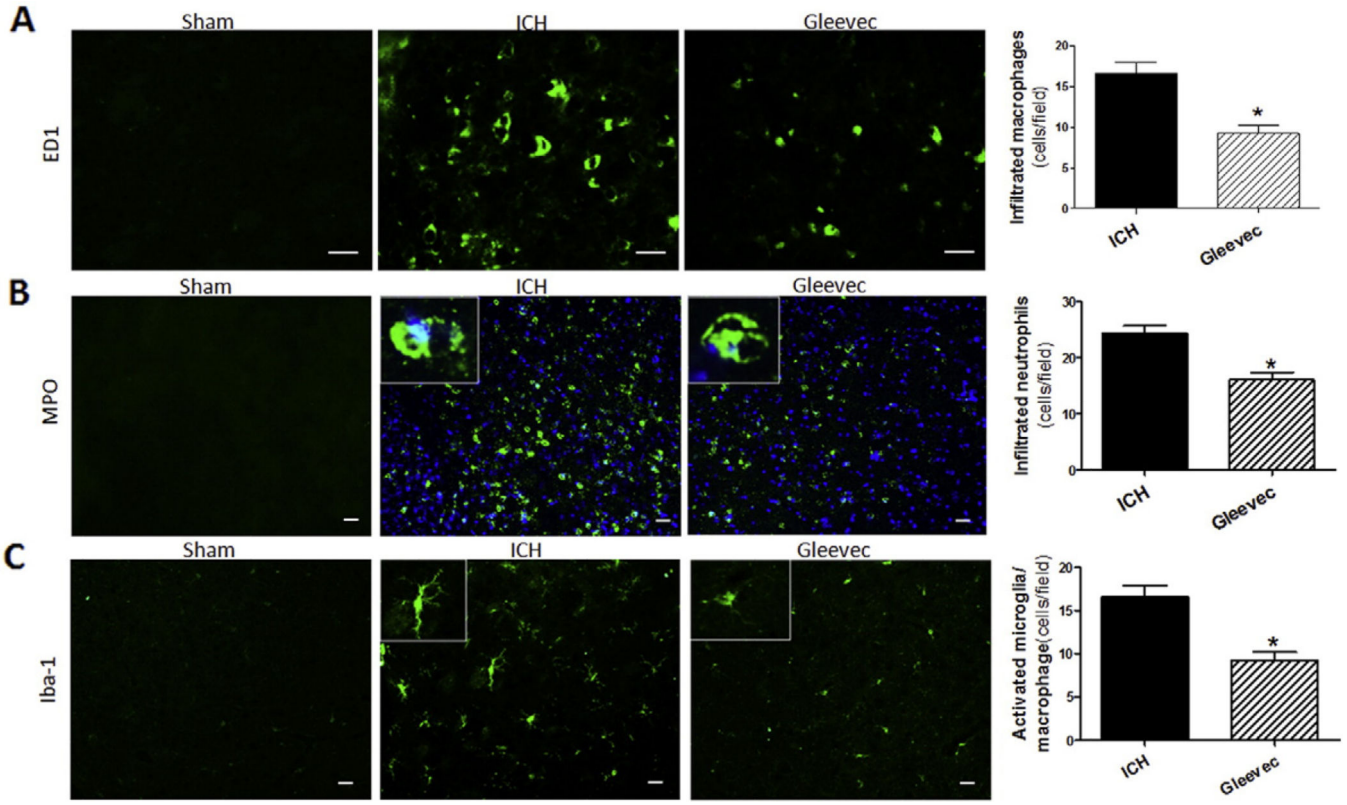

Fig. 3.

Gleevec inhibited macrophage infiltration in the perihematomal area 24 h after ICH. (A) Compared to shams, there was a robust increase in the number of macrophages that infiltrated the brain after ICH at 24 h. Gleevec significantly decreased the macrophage infiltration. (B) ICH resulted in brain infiltration by MPO positive neutrophils at 24 h that was significantly attenuated by Gleevec. (C) At 24 h ICH increased the number of activated microglia (Iba-1 staining) that was significantly reduced by Gleevec administration. Scale bars = 50um. *$p < 0.05$ vs ICH group, n= 6 mice per group.

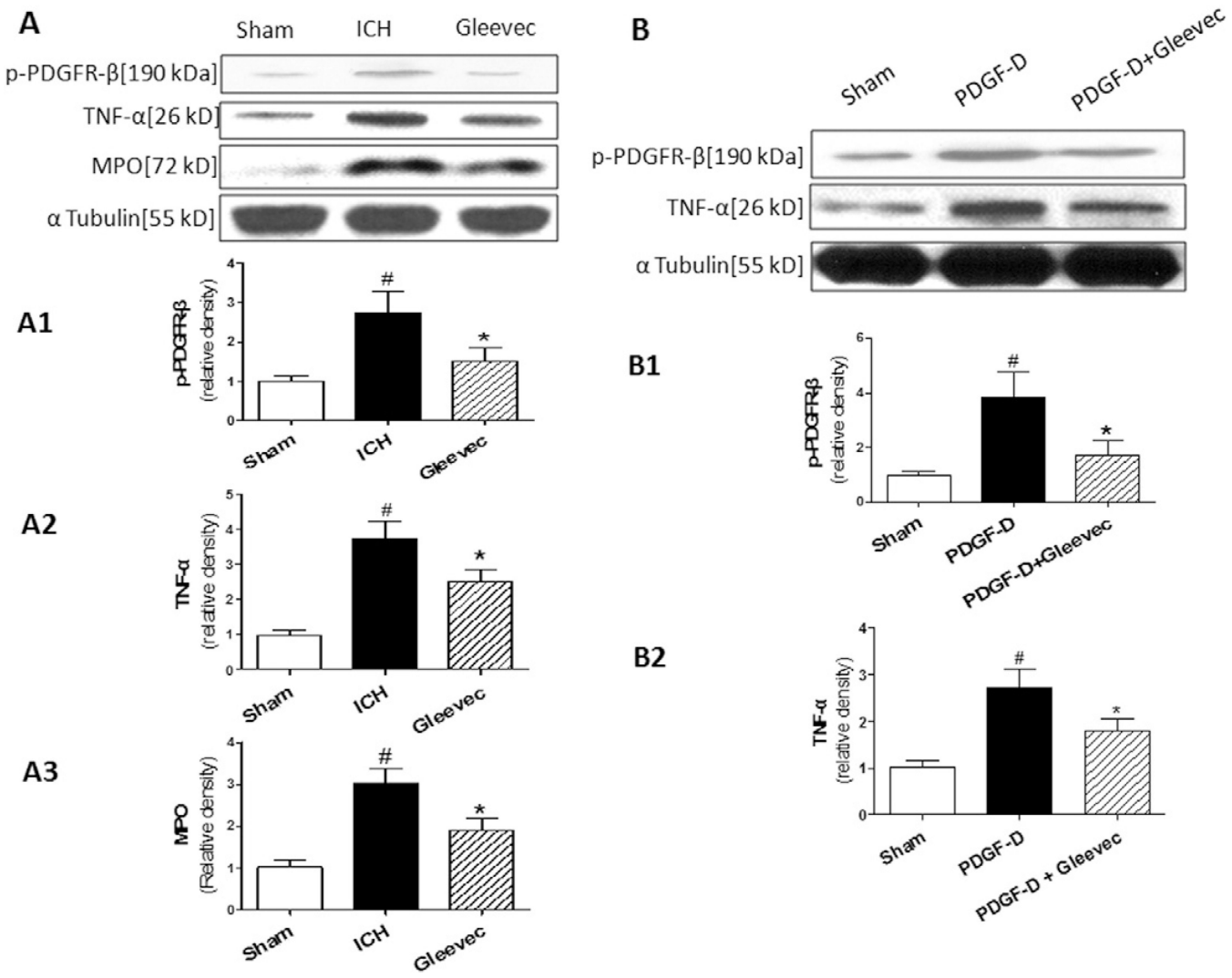

Fig. 4.

Gleevec suppressed ICH and PDFG-D induced inflammation. (A) A representative Western blot demonstrating that ICH increases expression of p-PDGFRβ (A1), TNF-α(A2) and MPO (A3) in the ipsilateral hemisphere of ICH compared to sham animals 24 h after ICH. Gleevec significantly ameliorated this effect. (B) Administration of the recombinant PDGF-D also induced p-PDGFRβ (B1) and TNF-α (B2) production in the brains of naïve animals that was reversed by Gleevec ($^{\#}p < 0.05$ vs sham; $^{*}p < 0.05$ vs Gleevec; n = 6 mice per group).

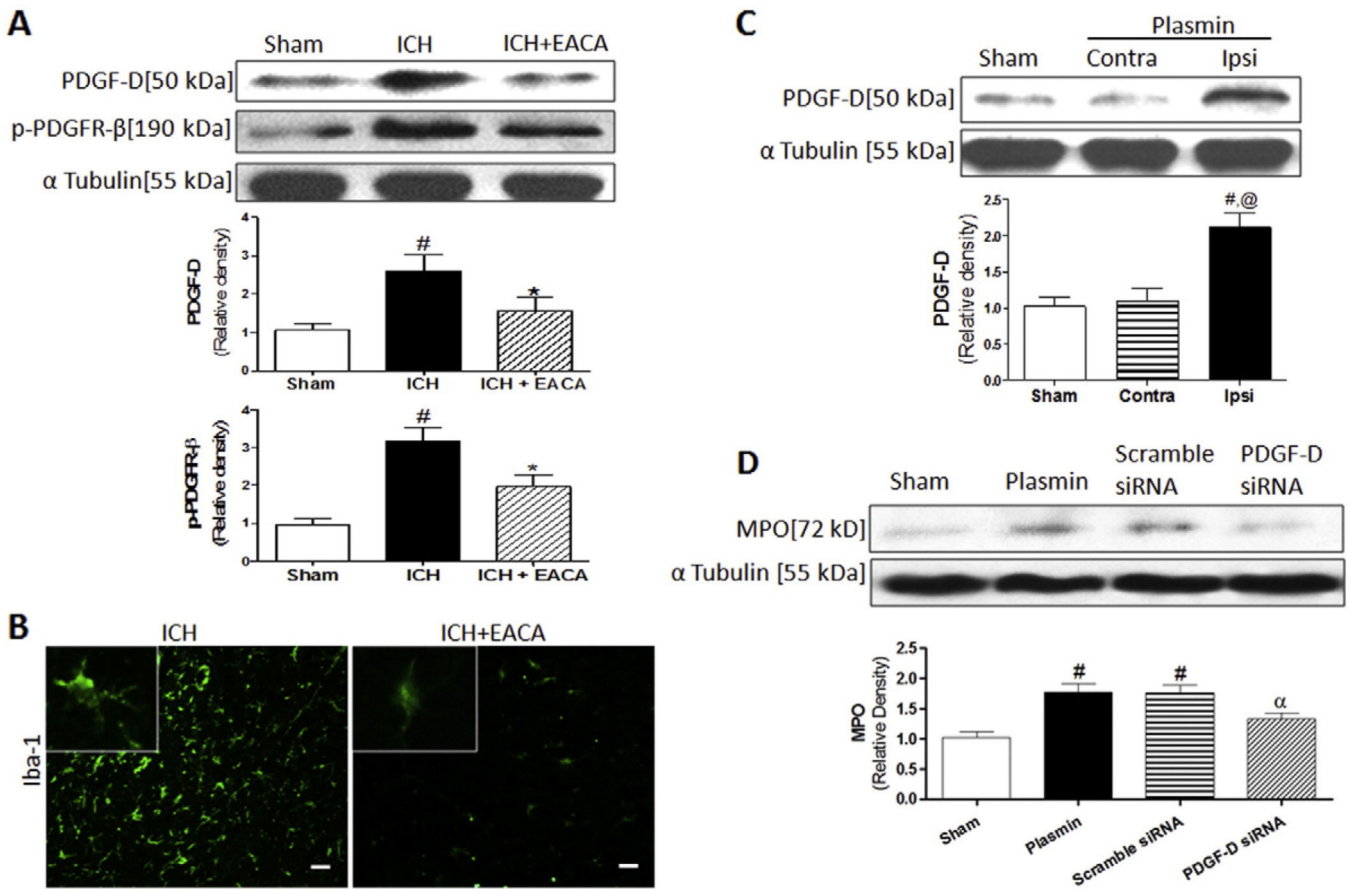

Fig. 5.

Effects of plasmin on PDGF-D induced inflammation. The plasmin antagonist (EACA) decreased ICH induced production of PDGF-D and p-PDGFRβ increases 24 h after ICH (A, $^{\#}p < 0.05$ vs sham. $^{*}p < 0.05$ vs ICH. n = 6 mice per group), resulting in decreased microglia activation (B). Injection of the recombinant plasmin induced significant production of the PDGF-D in ipsilateral hemisphere (C, $^{\#}p < 0.05$ vs sham, $^{@}p < 0.05$ vs contralateral. n = 6 mice per group). Increased MPO levels (D) were also observed after plasmin injection. While scrambled RNA has no effect, PDGF-D siRNA attenuated plasmin induced increase of MPO production (D, $^{\#}p < 0.05$ vs sham. $^{\alpha}p < 0.05$ vs Plasmin. n = 6 mice per group). Error bars represent mean ± standard error of the mean. Scale bar = 50um.