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Platelet-derived growth factor receptor- β regulates vascular smooth muscle cell phenotypic transformation and neuro-inflammation after intracerebral hemorrhage in mice

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Abstract

Objective—Platelet-derived growth factor-BB (PDGF-BB) activates platelet-derived growth factor receptor- β (PDGFR- β) and promotes vascular smooth muscle cell phenotypic transformation. Elevated levels of non-muscle myosin IIB (SMemb) are found in secretory smooth muscle cells along with inflammatory mediators, such as intercellular adhesion molecule-1

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(ICAM-1), which can amplify neutrophil infiltration into the brain. In the present study, we investigated the role of PDGF-BB/PDGFR- β following intracerebral hemorrhage (ICH)-induced brain injury in mice, with emphasis on its ability to promote vascular smooth muscle cell (VSMC) phenotypic transformation followed by increased ICAM-1 expression and elevated neutrophil infiltration in the vicinity of the hematoma. We also determined the extent to which plasmin from the hematoma influences the PDGF-BB/PDGFR- β system subsequent to ICH.

Methods—Brain injury was induced by autologous arterial blood (bICH) or plasmin injection into mouse brains. Small interfering RNA targeting PDGFR- β was administered 24 hours before ICH. A PDGFR antagonist, Gleevec, was administered following ICH. A mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2, MK2) inhibitor (KKKALNRQLGVAA) was delivered with PDGF-BB in naïve animals. PDGF-BB was injected with a plasmin inhibitor (ϵ -Aminocaproic acid, EACA) in ICH mice. Plasmin-injected mice were given PDGFR- β small interfering RNA 24 hours before the operation. Neurological deficits, brain edema, Western blots and immunofluorescence were evaluated.

Results—PDGFR- β siRNA-attenuated SMemb and ICAM-1 expression and neutrophil infiltration at 24 hours post-injury, and reduced neurological deficits and brain edema at 24 and 72 hours following ICH. The PDGFR antagonist, Gleevec, reduced SMemb and ICAM-1 expression. PDGFR- β activation led to increased expression of ICAM-1 and was reversed by KKKALNRQLGVAA in naïve mice. Plasmin inhibition suppressed PDGFR- β activation and neutrophil infiltration, whereas exogenous PDGF-BB increased PDGFR- β activation, regardless of plasmin inhibition. PDGFR- β siRNA decreased the expression of ICAM-1 by plasmin injection.

Interpretation—The PDGF-BB/PDGFR- β system contributes to neuro-inflammation through VSMC phenotypic transformation near the hematoma via the p38 MAPK/MK2 pathway following ICH. Plasmin is hypothesized to be upstream of the proposed neuro-inflammatory system. The therapeutic intervention targeting the PDGF-BB/PDGFR- β is a novel strategy to prevent plasmin-induced brain injury following ICH.

Keywords

Platelet-derived growth factor-BB; Platelet-derived growth factor receptor β ; vascular smooth muscle cell; phenotypic transformation; neuro-inflammation; intracerebral hemorrhage

INTRODUCTION

Spontaneous intracerebral hemorrhage (ICH) is an often fatal stroke subtype associated with inflammatory mechanisms that produce detrimental neurological injury.^{1,2} In current clinical practice, there is no effective therapy for this pattern of ICH.³ Following ICH, blood-derived leukocytes, mostly neutrophils, infiltrate into the brain parenchyma, activating resident microglia, enhancing the production of proinflammatory cytokines, and then likely contribute to deleterious neurological outcomes.^{4,5} Intercellular adhesion molecule-1 (ICAM-1) has been reported to be a potential factor facilitating increased neutrophil infiltration into the brain parenchyma after ICH.^{6,7} Vascular smooth muscle cells (VSMC) are key components of the vascular neural network and play an important role in stroke pathophysiology.⁸ VSMC marker proteins are commonly used to define the VSMC phenotype, whereas α -smooth muscle actin (α -SMA) is a typical marker protein of all

smooth muscle, and Non-Muscle Myosin IIB (SMemb) and ICAM-1 are the marker proteins for the VSMC synthetic phenotype.⁹ Additionally, accumulating evidence suggests that inflammatory transformation of VSMC can significantly increase the expression of other inflammatory mediators, such as ICAM-1.^{9,10,11} A relation between neuro-inflammation and VSMC phenotypic transformation is strongly suggested by multiple lines of evidence, and further research is necessary to understand the pathophysiological consequences of this dynamic. Overall, the effects of VSMC phenotypic transformation after ICH *in vivo* remain unclear.

Platelet-derived growth factor-BB (PDGF-BB) has been found to bind and activate platelet-derived growth factor receptor beta (PDGFR- β) *in vivo*.¹² Previous investigations have also reported that PDGF-BB is predominantly expressed in VSMC within the brain.^{12,13} Additional studies have indicated that PDGF-BB may act as a potent mediator of VSMC phenotypic modulation^{14,15,16} and may be involved in the pathophysiology of promoting ICAM-1 expression in VSMC.¹⁷ PDGFR- β , a tyrosine kinase receptor, is mainly expressed in VSMC and pericytes in the brain.^{12,13} Although a broad variety of research has demonstrated that PDGFR- β plays a key role in VSMC phenotypic transformation, the importance of this process in the ICH-induced injury remains unclear.^{9,18}

The present study investigated the role of the PDGF-BB/PDGFR- β pathway following ICH-induced VSMC phenotypic transformation in the vicinity of the hematoma with emphasis on the development of neuroinflammation through multiple pathways in an established blood injection ICH mouse model.

SUBJECTS/MATERIALS AND METHODS

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 12-hour light/dark cycles at a controlled temperature and humidity with free access to food and water.

ICH Mouse Model and Injection of Plasmin into Basal Ganglia

ICH was induced using the autologous arterial blood injection model (bICH), which was modified from previous descriptions.¹⁹ Mice were positioned in a stereotactic frame (Kopf Instruments, Tujunga, CA) after they were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Rectal temperature was maintained at 37.5°C using a feedback-controlled heating pad. A cranial burr hole (1 mm) was drilled on the right side of the skull (0.2 mm anterior and 2.0 mm lateral of the bregma). Autologous blood (30 μ l) was collected from tail arterial into a capillary tube, without heparin, and transferred into a 250 μ l Hamilton syringe which was then stereotactically inserted into the right basal ganglia through the burr hole. Either 30 μ l of autologous blood or 2 μ l of plasmin (Haematologic Technologies, 0.7ug/ μ l) was infused at 2 μ l/min by a microinjection pump. The needle was left in place for 10 minutes after injection to prevent reflux and then it was slowly removed. Following the surgery, the burr hole was sealed with bone wax; the incision

was closed with sutures. Animals were sacrificed for designated experiments at 3, 6, 12, 24, and 72 hours following ICH surgery.

Experimental Design

Six experiments were conducted (Fig 1, experiments 1–6) in our experimental design. More detailed descriptions are in supplemental materials.

Experiment 1: Thirty-six mice were divided into 6 groups (Sham, and 3, 6, 12, 24, 72 hours after ICH). Molecularly, the temporal expression of PDGF-BB and PDGFR- β was characterized by Western blot.

Experiment 2: The PDGFR- β siRNA mixture was administered intraventricularly 24 hours before ICH. Samples for Western blot and immunostaining were collected 24 hours after ICH. Neurological deficits and brain edema were measured at 24 and 72 hours.

Experiment 3: The PDGFR antagonist, Gleevec, was administered (intraperitoneal injection) 1 hour following ICH. Samples for Western blot and immunostaining were collected 24 hours after ICH.

Experiment 4: Recombinant PDGF-BB was injected with or without a MK2 inhibitor into right basal ganglia in naïve mice. Samples for Western blot and immunostaining were collected 24 hours after operation.

Experiment 5: The plasmin inhibitor, EACA, with or without recombinant PDGF-BB, was injected with blood into right basal ganglia. Western blot and immunostaining were conducted 24 hours following injection.

Experiment 6: The PDGFR- β siRNA was administered intraventricularly 24 hours before plasmin injected into right basal ganglia in naïve mice. Post assessment included Western blot and immunostaining.

Mice were randomly divided into these groups for above experiments: sham (n=24), ICH (n=26), ICH + scramble siRNA (n=26), ICH + PDGFR- β siRNA (n=26), ICH + Gleevec (n=6), Naïve + PDGF-BB (n=6), Naïve + PDGF-BB + MK2 inhibitor (n=6), ICH + EACA (n=12), ICH + EACA + PDGF-BB (n=6), Naïve + plasmin (n=6), Naïve + plasmin + scramble siRNA (n=6), Naïve + plasmin + PDGFR- β siRNA (n=6). No mortality was observed in this study.

PDGFR- β siRNA Injection

PDGFR- β (OriGene) siRNA mixtures or scramble siRNA, dissolved in sterile RNase free siRNA Duplex Resuspension Buffer, was intraventricularly injected as previously described.³⁵ Mice were anesthetized and positioned in the same manner as described in ICH modeling above. PDGFR- β siRNA mixture or scramble siRNA (100 pmol/2 μ l) was delivered into the ipsilateral ventricle with a Hamilton syringe over 2 min.

Neurobehavioral Function Test

Neurobehavioral functions were evaluated by an independent researcher blinded to the procedure by the Garcia test and Forelimb placement test.²⁰ In the Garcia test, seven items including spontaneous activity, axial sensation, vibrissae proprioception, symmetry of limb movement, lateral turning, forelimb walking and climbing were tested with a maximum neurological score able to be achieved at 21 (healthy animal). In the forelimb placement test, animals held by their trunk, were 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 number of successful forelimb placements out of 10 consecutive trials.

Brain Water Content Measurement

Brain water content was measured as previously described.²⁰ 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 $[(WW - DW)/WW] \times 100$.

Western Blotting

Western Blotting was performed as described previously.²² Animals were euthanized 24h after ICH. Intracardiac perfusion with cold phosphate-buffered saline (PBS, pH 7.4) solution was performed, followed by removal of the brain and separation into ipsilateral and contralateral cerebrums. The brain parts were stored appropriately at -80°C immediately until analysis. Protein extraction from whole-cell lysates of ipsilateral brain were obtained by gently homogenizing in RIPA lysis buffer (Santa Cruz) with phosphatase inhibitors (Sigma) with further 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). Equal amounts of protein were loaded on an SDS-PAGE gel. After being electrophoresed and transferred to a nitrocellulose membrane, the membrane was blocked and incubated with the primary antibody overnight at 4°C. The primary antibodies were: anti-PDGFR- β (1:1000, Santa Cruz), anti-p-PDGFR- β (1:1000, Santa Cruz), anti-PDGF-BB (1:1000, abcam), anti-p-p38 MAPK(1:1000, Santa Cruz), anti-p-MK2 (1:1000, Santa Cruz), anti-MPO (1:1000, Santa Cruz), anti-ICAM-1(1:1000, Santa Cruz), and anti-smooth muscle actin(1:1000, Santa Cruz), anti-SMem(1:1000, abcam). The nitrocellulose membranes were incubated with secondary antibodies (Santa Cruz) for 1 hour 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 was analyzed using Image J software.

Immunofluorescence

At 24 hours following ICH, mice were perfused under deep anesthesia with cold phosphate-buffered saline (PBS, pH 7.4), then infused with 10% formalin. Brains were removed and

fixed in formalin at 4°C for a minimum of 3 days. Samples were dehydrated with 30% sucrose in phosphate-buffered saline (PBS, pH 7.4) and the frozen coronal slices (10 µm thick) were sectioned in cryostat (CM3050S; Leica Microsystems). Immunofluorescence was performed as previously described.^{22,36} Anti-PDGFR-β antibody (1:100, Santa Cruz), anti-p-p38 MAPK (1:100, Santa Cruz), anti-p-MK2 (1:100, Santa Cruz) anti-ICAM-1(1:100, Santa Cruz), anti-SMem (1:100, abcam) were incubated separately with primary antibodies: anti-smooth muscle actin (1:1000, Santa Cruz) overnight at 4°C. It was then incubated with the appropriate fluorescence conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). The slices were observed underneath a fluorescence microscope (Olympus BX51, Olympus Optical Co. Ltd, Japan), and pictures were taken with software MagnaFire SP 2.1B (Olympus, Melville, NY).

Statistics

Data was expressed as mean ± standard error of the mean. The analysis of the data was performed using GraphPad Prism software. Statistical differences between 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 Student-Newman-Keuls test. Statistical significance was defined as $p < 0.05$.

RESULTS

PDGF-BB and PDGFR-β Were Upregulated Following ICH Injury

Western blot results indicated that PDGF-BB level was increased in the ipsilateral hemisphere (Ipsi) 24 hours post bICH when compared to contralateral hemisphere (Contra) ($p < 0.05$) and sham mice ($p < 0.05$; Fig 2 A1,A2). Western blots were performed to determine the profile of PDGFR-β in sham mice as well as 3, 6, 12, 24 and 72 hours after ICH. The results showed that PDGFR-β levels (Fig 2 B1,B2) were increased ($p < 0.05$) 6 hours post ICH and remained at the high levels until 24 hours ($p < 0.05$). The double immunofluorescence staining revealed that PDGFR-β immunoreactivity was found on VSMC around perihematoma area (Fig 2 C).

The VSMC phenotype converted to an inflammatory secreting state in the peri-hematoma area 24 hours post ICH, and PDGFR-β siRNA injection suppressed this phenomenon

The double immunofluorescence staining revealed SMemb and ICAM-1 immunoreactivity in VSMC (Fig 3 B) in the perihematoma area 24 hours after ICH. Western blot results for SMA levels of in the ipsilateral hemisphere demonstrated a decreasing trend, but the results were not statistically significant. SMemb and ICAM-1 levels increased significantly 24 hours after ICH in the ipsilateral hemisphere ($p < 0.05$; Fig 3 A). Additionally, the SMemb and ICAM-1 levels were attenuated by PDGFR-β siRNA injection compared to ICH, ICH +scramble siRNA animal groups ($p < 0.05$; Fig 3 A).

PDGFR-β siRNA injection suppressed the phosphorylation of PDGFR-β level followed by attenuation of the p38 MAPK/MK2 pathway post ICH

24 hours following ICH, Western blot results showed that phosphorylated PDGFR-β levels were increased when compared to sham mice (Fig 4 A; $p < 0.05$), whereas levels were

attenuated by PDGFR- β siRNA injection compared to ICH and ICH+scramble siRNA group ($p<.05$; Fig 4 A). PDGFR- β siRNA injection also reduced the levels of phosphorylated p38 MAPK and phosphorylated MK2 ($p<.05$; Fig 4 A) when compared to ICH or ICH+scramble siRNA groups. Additionally, the cellular localization of PDGFR- β downstream mediators were determined by double immunofluorescence staining. Similar to PDGFR- β , phosphorylated p38 and phosphorylated MK2 immunoreactivity were found in the VSMC (Fig 4 B).

PDGFR- β siRNA injection reduced neutrophil infiltration and brain edema as well as improved neurobehavioral functions

To investigate the effect of PDGFR- β on neutrophil infiltration, we tested myeloperoxidase (MPO) levels in the ipsilateral hemisphere by Western blot as well as immunostaining at 24 hours after ICH. Western blot results showed that MPO levels in the ipsilateral hemisphere significantly increased after ICH and were reduced after PDGFR- β siRNA injection when compared to ICH or ICH+scramble siRNA groups ($p<.05$; Fig 5 A). Representative pictures of immunostaining showed that PDGFR- β siRNA treatment reduced the number of MPO-positive cells in the perihematoma area compared to ICH or ICH+scramble siRNA mice ($p<.05$; Fig 5 B).

ICH mice had behavioral deficits compared to sham group in the Garcia test ($p<.05$; Fig 6). After PDGFR- β siRNA injection, the Garcia tests improved at both 24 and 72 hours compared to ICH or ICH+scramble siRNA mice ($p<.05$). Consistent with neurological improvement, brain edema in the ipsilateral basal ganglia (Ipsi-BG) following PDGFR- β siRNA injection was also reduced at both 24 hours (Ipsi-BG: $80.67\pm0.28\%$ vs. ICH, $81.73\pm0.39\%$, $p<.05$; vs. ICH+scramble siRNA, $82.68\pm0.35\%$, $p<.05$) and 72 hours (Ipsi-BG: $81.64\pm0.30\%$ vs. ICH, $83.17\pm0.24\%$, $p<.05$; vs. ICH+scramble siRNA, $81.87\pm0.36\%$, $p<.05$) compared to ICH or ICH+scramble siRNA group.

Gleevec, a PDGFR antagonist, attenuated VSMC phenotypic transformation to an inflammatory state in the peri-hematoma area

Western blot results indicated that Gleevec could reduce SMemb and ICAM-1 levels 24 hours after ICH in the ipsilateral hemisphere ($p<.05$; Fig 7) when compared to ICH animal groups.

Recombinant PDGF-BB promoted ICAM-1 expression, but was reversed by a MK2 inhibitor in naïve mice

At 24 hours following recombinant PDGF-BB injection to the brain, ICAM-1 levels increased in the ipsilateral hemisphere compared to sham mice ($p<.05$; Fig 8). A MK2 inhibitor, KKKALNRQLGVAA, was co-injected into the right basal ganglia with PDGF-BB, and 24 hours later, the level of ICAM-1 decreased in the ipsilateral hemisphere compared to mice injected with PDGF-BB ($p<.05$; Fig 8).

Plasmin inhibition attenuated neutrophil infiltration while suppressing PDGFR- β activation and PDGF-BB expression post ICH

Plasmin is an enzyme present in blood that degrades many blood plasma proteins, including fibrin clots. When ICH occurs, blood flows into the brain parenchyma and plasminogen is converted to plasmin. EACA, also called 6-aminocaproic acid or ϵ -amino-n-caproic acid, is an inhibitor of plasmin. EACA was co-injected with autologous arterial blood into the right basal ganglia of mice to inhibit the plasmin in this experiment. After 24 hours, the levels of PDGF-BB, phosphorylated PDGFR- β and MPO were reduced (Fig 9A) compared to ICH animals ($p < .05$). The representative pictures of immunostaining showed that plasmin inhibition attenuated neutrophil infiltration 24 hours after ICH (Fig 9B).

PDGFR- β activation reversed the protective effects of plasmin inhibition on neutrophil infiltration post ICH

The level of phosphorylated PDGFR- β increased when EACA and recombinant PDGF-BB were injected with blood into right basal ganglia compared to mice treated only with EACA ($p < .05$; Fig 10). We also found the level of MPO increased in the EACA+PDGF-BB group compared to the EACA group ($p < .05$; Fig 10).

Plasmin injection increased PDGF-BB expression, and PDGFR- β siRNA injection reduced plasmin induced ICAM-1 expression 24 hours after operation in naïve mice

The PDGF-BB level was increased in the ipsilateral hemisphere (Ipsi) 24 hours following plasmin induced brain injury when compared to contralateral hemisphere (Contra) and sham group ($p < .05$; Fig 11 A). 24 hours after plasmin injection into the brain, the level of ICAM-1 increased in the ipsilateral hemisphere compared to that of sham mice ($p < .05$; Fig 11 B). In PDGFR- β siRNA injected animals, we found that the level of ICAM-1 decreased in the ipsilateral hemisphere compared to mice injected with plasmin only ($p < .05$; Fig 11 B).

DISCUSSION

We hypothesize that the PDGF-BB/PDGFR- β system may enhance the VSMC phenotypic transformation and amplify neuroinflammation via the p38 mitogen-activated protein kinase (p38 MAPK)/mitogen-activated protein kinase-activated protein kinase-2 (MK2) pathway. From this perspective, plasmin is a possible upstream activator of the PDGF-BB pathway that acts to increase the number of inflammatory cells that infiltrate through the blood brain barrier (BBB) *in vivo*. To test this hypothesis, we first investigated the expression profiles of PDGF-BB and PDGFR- β in the brain following ICH. We next applied PDGFR- β small interfering RNA (PDGFR- β siRNA) to inhibit PDGFR- β activation, and measured the phosphorylated level of the PDGFR- β while measuring multiple outcomes including VSMC phenotypic transformation and neuroinflammatory cell infiltration in the vicinity of the hematoma. Also, we used an antagonist for PDGFR, Gleevec, to test its ability to attenuate VSMC phenotypic transformation. Additionally, we gave an MK2 inhibitor to reverse the ICAM-1 expression that was exacerbated by PDGFR- β activation. To test whether plasmin may be an upstream mediator, ϵ -Aminocaproic acid (EACA), a plasmin inhibitor, was administered to animals with or without PDGF-BB co-injection following ICH. Finally,

PDGFR- β siRNA was introduced in an established plasmin injection model to determine the role of plasmin in activating and/or inhibiting the PDGF-BB/PDGFR- β pathway.

ICH is an often fatal stroke subtype associated with inflammatory mechanisms that precipitate widespread neurological injury.^{1,2} However, the mechanisms of the inflammatory response after ICH remains to be elucidated. In the present study, we made the following main observations: 1) Therapeutic interventions inhibiting the PDGF-BB/PDGFR- β system may be a novel strategy to attenuate VSMC phenotype transformation to an inflammatory state around the hematoma through the p38 MAPK/MK2 pathway following ICH injury, followed by reducing ICAM-1 expression and neutrophil infiltration into the brain. 2), Plasmin may be an essential upstream regulator of PDGF-BB/PDGFR- β system during hematoma formation following ICH.

Following ICH, accumulating evidence suggests that blood-derived leukocytes, the majority of which are neutrophils, infiltrate into the brain parenchyma, initiate the events in ICH, and enhance the disruption of the blood brain barrier, increasing brain edema and producing deterioration in neurobehavioral function.^{4,5} ICAM-1, typically expressed on vascular and immune system cells, induced proinflammatory effects, such as amplifying leukocyte recruitment signals and enhancing neutrophil infiltration into the brain, which exacerbates the brain injury in ICH models.^{6,7,21} The main function of VSMCs is to regulate tissue perfusion through graded contraction. Importantly, VSMC display a remarkable plasticity by converting among different phenotypes during some pathologies, a process referred to as VSMC phenotypic transformation.^{9,10} Emerging evidence indicates that when the VSMC phenotype modulates into an inflammatory state, expression of inflammatory mediators, such as ICAM-1, significantly increase.^{9,10,11,33}

What causes VSMC phenotypic transformation following ICH? What signals stimulate VSMCs near the hematoma to transform their phenotype after ICH? Recently reported data suggests that PDGFR- β activation and PDGF-BB are primarily responsible for VSMC phenotypic switching in cardiovascular system.^{14,15,16,18} Additionally, a number of studies have shown that inflammatory cytokines, such as tumor necrosis factor alpha (TNF- α) and interleukin (IL)-1, promote the expression of ICAM-1, leading to the conversion of VSMC to an inflammatory phenotype capable of facilitating the infiltration of inflammatory cells.^{9,10,11} Extracellular levels of TNF- α and IL-1 can increase around the hematoma after ICH⁴, and the expression of PDGFR- β can be increased at 6 hours after ICH and maintained at elevated levels for 24 hours with a subsequent decline at 72 hours. In the present studies, the PDGF-BB levels were much greater in the ipsilateral hemisphere than in the contralateral hemisphere at 24 hours after ICH, supporting the hypothesis that near the hematoma, PDGF-BB contributes to VSMC phenotypic transformation to an inflammatory state. In further support of this idea, our Western blot results revealed that the level of SMemb and ICAM-1 increased in the ipsilateral hemisphere 24 hours after ICH. Immunofluorescence results also demonstrated that the SMemb and ICAM-1 positive cells co-localized with VSMCs near the hematoma, suggesting that VSMCs surrounding the hematoma converted to an inflammatory, secretory phenotype. VSMC phenotypic switching was attenuated after administration of PDGFR- β siRNA, indicating that PDGFR- β facilitates the phenotypic transformation of VSMC near the hematoma. Western blots and

immunofluorescence of MPO further indicated that the increase of ICAM-1 was associated with increased recruitment of neutrophils into the brain parenchyma at 24 hours after ICH, and was reversed after administration of PDGFR- β siRNA. These recruited neutrophils probably contribute to the increased brain edema and neurological deficits observed at 24 and 72 hours following ICH.

To explore the potential clinical significance of our study, we examined the effect of Gleevec, a PDGFR antagonist, on attenuating VSMC phenotypic transformation. Gleevec is an anti-cancer drug attributed with BBB protective effects at a dose of 60 mg/kg.²² We found that Gleevec reduced SMemb and ICAM-1 levels 24 hours after ICH in the ipsilateral hemisphere when compared to untreated ICH animals. These results were consistent with effects of the PDGFR- β siRNA injections.

Although some concern might be raised about why we did not observe decreases in SM α A after ICH, we attribute this to preservation of the existing smooth muscle. Virtually all smooth muscle cells express SM α A, even when in a migratory, proliferative, or synthetic phenotype¹⁸. In addition, SMemb and ICAM-1 positive cells co-localized within the VSMCs near the hematoma, indicating phenotypic conversion to an inflammatory state at 24 hours after ICH. Not all VSMCs will exhibit phenotypic transformation at 24 hours after ICH, but all will express SM α A. Given this biology, it is expected that the level of SM α A in the ipsilateral hemisphere remained relatively unchanged.

The potential signaling downstream of PDGF-BB/PDGFR- β may explain the mediation of VSMC phenotypic transformation. The p38 MAPK pathway has been established as a downstream effector of PDGFR- β signaling in VSMC.¹⁰ In addition, p38 MAPK can modulate ICAM-1 expression in VSMC.^{23,24} Activation of p38 leads to phosphorylation of MK2, which can directly regulate ICAM expression²⁵. Correspondingly, MK2 deficiency attenuates phenotypic transformation of VSMC after hemorrhagic injury.²⁶ But, so far, there is no similar findings in ICH model. In our study, we reported that phosphorylated PDGFR- β , phosphorylated p38 MAPK, phosphorylated MK2, SMemb, and ICAM-1 levels were all increased 24 hours after ICH. Additionally, administration of recombinant PDGF-BB promoted ICAM-1 expression, which was reversed after the use of a MK2 inhibitor in naïve mice. Moreover, we found the phosphorylated PDGFR- β , phosphorylated p38 MAPK, and phosphorylated MK2 were all expressed in VSMCs in the peri-hematoma area 24 hours following ICH. These findings suggest that the PDGF-BB/PDGFR- β system may orchestrate the VSMC phenotypic transformation to an inflammatory state through p38 MAPK/MK2 signaling.

The question then arises as to what is the upstream initiator of PDGF-BB/PDGFR- β induced VSMC phenotypic transformation? PDGF-BB can specifically bind to and activate PDGFR- β *in vivo*.¹² Many growth factors exist in latent forms, and their activation often requires proteolytic processing.²⁷ An *in vitro* study demonstrated that incubation with plasmin caused an increase in the release of PDGF-BB.²⁸ In addition, plasmin might be the major protease responsible for processing PDGF.²⁹ After ICH, coagulation is initiated by the activation of thrombin, followed by fibrin formation. Plasmin cleaves fibrin, resulting in clot lysis and generation of fibrin fragments, followed by modulation of the inflammatory

response by affecting leukocyte migration and cytokine production.^{30,31} Plasmin was also found to play a deleterious role in neuro-inflammation and injury to the blood brain barrier and brain parenchyma *in vivo*.^{4,32,34} Therefore, in the present study, we investigated the potential relationship between plasmin and the PDGF-BB/PDGFR- β system. First, the plasmin inhibitor, EACA, was co-injected with autologous arterial blood into the right basal ganglia of mice. We found that EACA attenuated neutrophil infiltration, while suppressing PDGFR- β activation and PDGF-BB expression post ICH. Recombinant PDGF-BB reversed the protective effects of plasmin inhibition. Additionally, in the plasmin induced brain injury, plasmin injection increased PDGF-BB expression, while PDGFR- β siRNA injection reduced plasmin induced ICAM-1 increasing 24 hours after operation in naïve mice. Taken together, these findings demonstrate that plasmin is potentially an essential upstream regulator of PDGF-BB/PDGFR- β system.

Among the limitations in this study, direct evidence of VSMC phenotypic switching obtained by measurements of smooth muscle contractility, myofilament Ca^{2+} sensitivity, or other related techniques has not been provided. In future studies, we will separate the vasculature from the brain and complete these measurements. In addition, endothelial cells also have phenotypic transformation¹⁸ and may be involved in immune cell infiltration and inflammation. Endothelial cells are not studied due to the scope of the current study. In addition, the importance of PDGFR- β expressed in pericytes after ICH will be investigated.

In conclusion, our findings suggest that PDGF-BB/PDGFR- β may contribute to neuro-inflammation through enhancing VSMC phenotypic transformation to an inflammatory state via p38 MAPK/MK2 pathway in the vicinity of the hematoma following ICH. In addition, plasmin was shown to be an upstream initiator of the PDGF-BB/PDGFR- β cascade. Given the lack of treatment options for ICH, targeting PDGF-BB/PDGFR- β signaling may provide a novel treatment for neuro-inflammation in this pathophysiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1. Experimental design and animal group classification

ICH = intracerebral hemorrhage; VSMC = vascular smooth muscle cell; PDGF-BB = platelet-derived growth factor BB; PDGFR- β = platelet-derived growth factor receptor β ; MK2 = mitogen-activated protein kinase-2; ICAM-1 = intercellular adhesion molecule-1; EACA = ϵ -Aminocaproic acid (a plasmin antagonist); ICV = intraventricular injection; ip = intraperitoneal injection; WB = western blotting; IF = immunofluorescence.

FIGURE 2. PDGF-BB and PDGFR- β Were Upregulated Following ICH Injury

(A1) Western blot assay for PDGF-BB expression in Sham, ipsilateral hemisphere (Ipsi) and contralateral hemisphere (Contra) 24 hours after ICH. (B1) Western blot assay for the profiles of PDGFR- β expression in the ipsilateral hemisphere of sham and ICH mice at 3, 6, 12, 24, and 72 hours after operation. (C) Represented photographs of immunofluorescence staining for PDGFR- β (red) expression in vascular smooth muscle cells (α -SMA, green) in the perihematoma area 24 hours after ICH. Bar=50 μ m. Quantification of A1 and B1 is shown in A2 and B2 separately; n=6 mice per group and per time point. Error bars represent mean $\pm$ standard error of the mean. # p<0.05 vs Sham; @ p<0.05 vs Contra.

FIGURE 3. The VSMC phenotype switching to an inflammatory secreting state in the perihematoma area 24 hours post ICH while PDGFR-β siRNA injection suppress this phenomenon (A) Western blot assay for the expression of α-SMA, SMemb and ICAM-1 in the ipsilateral hemisphere in Sham, ICH, ICH+scramble siRNA, and ICH+PDGFR-β siRNA groups at 24 hours following operation. (B) Representative photographs of immunofluorescence staining for Smemb (red), ICAM-1 (red) with VSMC (α-SMA, green) in the perihematoma area 24 hours after ICH. Bar=50um. Quantification of A is shown in A1, A2 and A3 separately; n=6 mice per group. Error bars represent mean ± standard error of the mean. # p<0.05 vs sham, * p<0.05 vs ICH, @ p<0.05 vs ICH+scramble siRNA.

FIGURE 4. PDGFR-β siRNA injection suppressed the phosphorylation of PDGFR-β level followed by attenuating the p38 MAPK/MK2 pathway post ICH
 (A) Western blot assay for the expression of phosphorylated PDGFR-β (p-PDGFR-β), phosphorylated p38 (p-p38) and phosphorylated MK2 (p-MK2) in the ipsilateral hemisphere in Sham, ICH, ICH+scramble siRNA, and ICH+PDGFR-β siRNA groups at 24 hours following operation. (B) Representative photographs of immunofluorescence staining for p-p38 (red) and p-MK2 (red) with VSMC (α-SMA, green) in the perihematoma area 24 hours after bICH. Bar=50um. Quantification of A is shown in A1, A2 and A3 separately; n=6 mice per group. Error bars represent mean ± standard error of the mean. # p<0.05 vs sham, * p<0.05 vs ICH, @ p<0.05 vs ICH+scramble siRNA.

FIGURE 5. Neutrophil infiltration surround the hematoma

(A,A1) Western blot assay for the myeloperoxidase (MPO) level in the ipsilateral hemisphere in Sham, ICH, ICH+scramble siRNA, ICH+PDGFR-β siRNA groups at 24 hours following operation; n=6 mice per group. Error bars represent mean standard error of the mean. * p<0.05 vs sham; # p<0.05 vs ICH; @ p<0.05 vs ICH+scramble siRNA.(B) Representative photographs of immunofluorescence staining for MPO-positive cells (green) in the perihematoma area in Sham, ICH, ICH+scramble siRNA and ICH+PDGFR-β siRNA groups 24 hours following ICH. Scale bar=50um.(B1) The quantification of MPO-positive cells in the perihematoma areas in ICH, ICH+scramble siRNA and ICH+PDGFR-β siRNA groups at 24 hours following operation(12 fields/brain);n=6 mice per group. Error bars represent mean +/- standard error of the mean. * p<0.05 vs ICH, @ p < 0.05 vs ICH +PDGFR-β siRNA.

FIGURE 6. PDGFR-β siRNA injection improved neurological functions and reduced brain edema at 24 and 72 hours following ICH

(A) Garcia test at 24 and 72 hours following operation in sham, ICH, ICH+scramble siRNA and ICH+PDGFR-β siRNA groups. (B) Forelimb Placement test at 24 and 72 hours following operation in sham, ICH, ICH+scramble siRNA and ICH+PDGFR-β siRNA groups. (C,D) Brain edema at 24 hours (C) and 72 hours (D) following operation in sham, ICH, ICH+scramble siRNA and ICH+PDGFR-β siRNA groups. Brain sections (4mm) were divided into 4 parts: ipsilateral basal ganglia (Ipsi-BG), ipsilateral cortex (Ipsi-CX), contralateral basal ganglia (Cont-BG), and contralateral cortex (Cont-CX). Cerebellum (Cerebel) is the internal control; n=6–13 mice per group. Error bars represent mean ± standard error of the mean. # p<0.05 vs sham, * p<0.05 vs ICH, @ p<0.05 vs ICH+scramble siRNA.

FIGURE 7. Gleevec, a PDGFR antagonist, attenuated VSMC phenotype switching to an inflammatory state in the peri-hematoma area

(A) Western blot assay for the expression of SMemb and ICAM-1 in the ipsilateral hemisphere in Sham, ICH, Gleevec groups at 24 hours following operation. Quantification of A is shown in B; n=6 mice per group. Error bars represent mean $\pm$ standard error of the mean. # p<0.05 vs sham, * p<0.05 vs ICH.

FIGURE 8. Recombinant PDGF-BB promoted ICAM-1 expression but was reversed when using a MK2 inhibitor in naïve mice

(A) Western blot of the ICAM-1 expression in the ipsilateral hemisphere at 24 hours after Sham, PDGF-BB and PDGF-BB+MK2 inhibitor injection in naïve mice; (B) Quantification of A is shown in B. n=6 mice per group. Error bars represent mean $\pm$ standard error of the mean. # p<0.05 vs Sham; * p<0.05 vs PDGF-BB.

FIGURE 9. Plasmin inhibition attenuated neutrophil infiltration while suppressing PDGFR- β activation and PDGF-BB expression 24 hours post ICH

(A) Western blot result of the PDGF-BB, p-PDGFR- β and MPO expression in the ipsilateral hemisphere 24 hours after operation of sham, ICH and ICH+EACA group. (B)

Representative photos of immunofluorescence staining for MPO positive cells (green) in the peri-hematoma area of sham, ICH and ICH+EACA group. Bar=50 μ m. Quantification of A are shown in A1, A2 and A3. n=6 mice per group. Error bars represent mean $\pm$ standard error of the mean. [#] p<0.05 vs sham, ^{*} p<0.05 vs ICH.

FIGURE 10. PDGFR- β activation reversed the protective effects of plasmin inhibition on neutrophil infiltration post ICH

EACA, a plasmin inhibitor, with or without PDGF-BB was co-injected with autologous arterial blood. (A) Western blot assay for phosphor-PDGFR- β and MPO level in the ipsilateral hemisphere 24 hours after ICH in EACA and EACA+PDGF-BB mice. Quantification of A is shown in B. $n=6$ mice per group. Error bars represent mean $\pm$ standard error of the mean. $p < 0.05$ vs. EACA.

FIGURE 11. Plasmin injection increased PDGF-BB expression and PDGFR- β siRNA injection reduced plasmin induced ICAM-1 increasing 24 hours after operation in naïve mice (A1) Western blot of the PDGF-BB expression in the ipsilateral hemisphere (Ipsi) and contralateral hemisphere (Contra) 24 hours after plasmin injection in the naïve animals as well as sham mice. (B1) Western blot of the ICAM-1 expression in the Sham, plasmin, plasmin+scramble siRNA and plasmin+PDGFR- β siRNA group 24 hours after operation. Quantification of A1 and B1 are shown in A2 and B2. $n=6$ mice per group. Error bars represent mean $\pm$ standard error of the mean. # $p<0.05$ vs Sham; * $p<0.05$ vs Plasmin; @ $p<0.05$ vs Contra. & $p<0.05$ vs plasmin+scramble siRNA.