Published in final edited form as:

J Neurosci Res. 2015 January ; 93(1): 94–103. doi:10.1002/jnr.23475.

Imatinib Preserves Blood-Brain Barrier Integrity Following Experimental Subarachnoid Hemorrhage in Rats

Yan Zhan^{#1,2}, Paul R. Krafft^{#1}, Tim Lekic¹, Qingyi Ma¹, Rhonda Souvenir¹, John H. Zhang^{1,3}, and Jiping Tang¹

¹Department of Basic Sciences, Division of Physiology, Loma Linda University School of Medicine, Loma Linda, California, USA

²Department of Neurosurgery, the First Affiliated Hospital of Chongqing Medical University, Chongqing, China

³Department of Neurosurgery, Loma Linda University School of Medicine, Loma Linda, California, USA

These authors contributed equally to this work.

Abstract

Blood-brain barrier (BBB) disruption and consequent edema formation contributes to the development of early brain injury following subarachnoid hemorrhage (SAH). Various cerebrovascular insults result in increased platelet derived growth factor receptor alpha (PDGFR- α) stimulation, which has been linked to BBB breakdown and edema formation. This current study examined whether imatinib, a PDGFR inhibitor, can preserve BBB integrity in a rat endovascular perforation SAH model. Imatinib (40mg/kg or 120mg/kg), or its vehicle, were administered intraperitoneally at 1 hour after SAH induction. BBB leakage, brain edema, and neurological deficits were evaluated. Total and phosphorylated protein expressions of PDGFR- α , c-Src, JNK, and c-Jun were measured; and enzymatic activities of matrix metalloproteinase (MMP)-2 and MMP-9 were determined in the injured brain. Imatinib treatment significantly ameliorated BBB leakage and edema formation 24 hours following SAH, which was paralleled by improved neurological functions. Decreased brain expressions of phosphorylated PDGFR- α , c-Src, JNK, and c-Jun; as well as reduced MMP-9 activities were found in treated animals. PDGFR- α inhibition preserved BBB integrity following experimental SAH; however, the protective mechanisms remain to be elucidated. Targeting PDGFR- α signaling may be an advantageous strategy to ameliorate early brain injury following SAH.

Keywords

Subarachnoid hemorrhage; imatinib; blood-brain barrier; brain edema; rats

Corresponding author: Jiping Tang, MD, Department of Basic Sciences, Division of Physiology, Loma Linda University School of Medicine, Loma Linda, California 92354, USA, Phone: 909-558-4480 Fax: 909-558-4035, jtang@llu.edu.

Conflict of Interest

There is no conflict of interest to declare.

Introduction

Subarachnoid hemorrhage (SAH) refers to an intracranial bleed located between the pial and arachnoid membranes. This stroke subtype's aggregate incidence is 10.5 cases per 100,000 person-years (Linn et al. 1996). Moreover, SAH accounts for approximately 27% of all stroke-related years of potential life lost before the age of 65 (Johnston et al. 1998). Despite advances in diagnosis and surgical management, more than 46% of SAH survivors suffer lasting disabilities such as impaired cognitive, visuospatial, language, and sensorimotor functions (Kreiter et al. 2002; Suarez et al. 2006).

The development of early brain injury (EBI) and delayed cerebral vasospasm (CVS) are believed to be responsible for the poor clinical outcome following SAH. EBI comprises acute events such as hemorrhage-induced elevation of intracranial pressure, disruption of the blood-brain barrier (BBB), edema formation, as well as activation of inflammatory and cell death pathways (Fujii et al. 2013; Ostrowski et al. 2006). Prolonged contraction of vascular smooth muscle cells, resulting in CVS, occurs around the third day after SAH and may last for 2-3 weeks after the ictus (Wilkins 1990). However, pharmacological reduction of moderate and severe CVS failed to demonstrate significant long-term improvements in SAH patients (Macdonald et al. 2011; Macdonald et al. 2008). This discovery accentuated the necessity for innovative therapeutics targeting alternative SAH-induced pathogenic processes, including EBI.

Platelet-derived growth factor receptors (PDGFRs) - members of the class III subfamily of receptor tyrosine kinases - are ubiquitously expressed in the mammalian brain and play a role in the progression of central nervous system disease. Indeed, PDGFR signaling has been shown to aggravate BBB disruption during ischemic stroke - if tissue plasminogen activator therapy was initiated (Su et al. 2008). Moreover, PDGFR stimulation facilitated edema formation after experimental intracerebral hemorrhage (Ma et al. 2011), as well as development of vasospasm in SAH patients and preclinical rodent models (Gaetani et al. 1997; Shiba et al. 2012). However, the role of PDGFR, particularly PDGFR- α , in the development of EBI following SAH has not been intensively investigated. Therefore, we hypothesize PDGFR- α contributes to BBB impairment via the JNK/c-Jun cascade, mediating matrix metalloproteinase (MMP) activation following SAH. We also hypothesize that treatment with PDGFR inhibitor imatinib mesylate ameliorates BBB breakdown and neurofunctional deficits in a rat endovascular perforation SAH model.

Materials and Methods

Experimental animals

All experimental procedures designed and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory animals, were approved by the Institutional Animal Care and Use Committee (IACUC) at Loma Linda University. One hundred and forty-four male Sprague-Dawley rats (weighing 280-350g; Harlan, Indianapolis, IN) were subjected to either sham operation (n=33) or SAH induction (n=111). All animals were housed in a light- and temperature-controlled environment with free access to food and water.

SAH modeling and study design

The endovascular perforation SAH model was performed as previously described (Schwartz et al. 2000). Briefly, anesthetized rats were intubated and artificially ventilated with 3% isoflurane in a 70/30% medical air/oxygen gas mixture. With the animal in a supine position, the rodent's left external carotid artery (ECA) was exposed and a sharpened 4-0 monofilament nylon suture was inserted into the ECA. The suture was gently advanced into the internal carotid artery (ICA) until resistance was felt by the bifurcation of the anterior (ACA) and middle cerebral artery (MCA). Endovascular perforation was achieved by further advancing the suture 3mm past the point of resistance, followed by immediate withdrawal of the suture to allow reperfusion of the ICA. The sham operation consisted of suture insertion; however, no vessel perforation was performed.

The PDGFR inhibitor, imatinib mesylate (Gleevec®, Novartis) was dissolved in PBS and administered intraperitoneally in two different dosages (40 or 120mg/kg) 1 hour after SAH induction. Vehicle animals received PBS only.

One hundred and ten rats survived the surgery and passed all inclusion criteria (see below). These animals were randomly divided into the following groups: sham-operated (Sham; n=33) and SAH animals receiving either PBS administration (Vehicle; n=38), 40mg/kg imatinib (Imatinib-40mg; n=6), or 120mg/kg imatinib (Imatinib-120mg; n=33). Evans blue extravasation, Western blot, immunoprecipitation, and gelatin zymography assays were conducted at 24 hours; neurological function, body weight loss, and brain edema were evaluated at 24 and 72 hours after surgery.

SAH grading

At the time of euthanasia a photograph of the rat's brain base was taken and divided into 6 predetermined areas, as previously described (Sugawara et al. 2008). Each area was given a score from 0 to 3, depending on the amount of blood present. The total SAH grade was calculated by adding all area scores (maximum SAH grade=18). Animals presenting with mild SAHs (grade<9) or with coexisting subdural or epidural hemorrhages were excluded from this study.

Neurological function testing

Immediately prior to euthanasia, neurological function was evaluated in a blinded fashion, by means of the modified Garcia score (Garcia et al. 1995). This composite sensorimotor assessment consists of 6 sub-tests, evaluating the animal's spontaneous activity, its reaction to side stroking and vibrissae touch, as well as limb symmetry, forelimb walking, and climbing ability. Each sub-test received a score between 0 and 3 and the sum of all sub-tests was calculated to determine neurological function (maximal score of 18 for best test performance).

Blood-brain barrier permeability

Permeability of the BBB was evaluated by means of Evans blue extravasation assays, as previously described (Uyama et al. 1988). Briefly, anesthetized rats were subjected to Evans blue dye injection (2%; 5ml/kg) into the left femoral vein at 23 hours after SAH induction or

sham surgery. Deeply anesthetized rats were euthanized by transcardiac PBS perfusion and brain specimens were removed and separated into ipsi- and contralateral brain hemispheres. The amount of extravasated albumin-bound Evans blue dye was measured spectrophotometrically (Genesis 10uv Scanning; Thermo Electron Corporation, Madison, WI) at 615nm.

Brain water content

Brain edema (brain water content) was evaluated via the wet weight/dry weight method as previously described (Duris et al. 2011a). Briefly, rats were decapitated under deep isoflurane anesthesia; brains were rapidly collected, and divided into the hemispheres, cerebellum, and brain stem. All specimens were immediately weighed to obtain the wet weight (WW), and once again after dehydrating the samples for 72 hours at 105°C (dry weight, DW). The brain water content for each specimen was calculated as $[(WW - DW)/WW] \times 100(\%)$.

Western blotting and immunoprecipitation

Left hemispheric brain specimens were prepared for Western blot analysis as previously described (Suzuki et al. 2010). Equal amounts of protein (30µg/sample) were electrophoresed on SDS-PAGE gels and transferred onto nitrocellulose membranes, which were first blocked and then incubated overnight at 4°C with the following primary antibodies: anti-phospho-JNK, Thr183, Tyr185 (1:2000), anti-JNK (1:1000) antibodies purchased from Cell Signaling Technology and anti-phospho-c-Src, Tyr419 (1:1000), anti-c-Src (1:1000), anti-phospho-c-Jun, Ser63/73 (1:1000), anti-c-Jun (1:1000) as well as anti-β-actin (1:5000, loading control) antibodies obtained from Santa Cruz Biotechnology, Inc. Following that, the nitrocellulose membranes were incubated with the appropriate secondary antibodies (1:2000; Santa Cruz Biotechnology, Inc) for 1 hour at room temperature. Immunoblots were visualized with an ECL Plus chemiluminescence reagent kit (Amersham Bioscience, Arlington Heights, IL) and densitometrically quantified using the software ImageJ (NIH).

Immunoprecipitation of the PDGFR-α was conducted as previously described (Ma et al. 2011). Briefly, protein specimens were incubated overnight with anti-PDGFR-α antibody-immunoprecipitation matrix complex (Santa Cruz Biotechnology, Inc) at 4°C. Next, samples were centrifuged for 5 minutes (1000g, 4°C) and re-suspended in equal amounts of loading buffer. The precipitated protein samples were then electrophoresed on SDS-PAGE gels and probed with the phosphotyrosine-specific antibody, PY-99 (1:500, Santa Cruz Biotechnology, Inc) as well as with the unphosphorylated anti-PDGFR-α antibody (1:1000; Cell Signaling Technology) in order to determine the p-PDGFR-α/PDGFR-α ratio. Incubation with the specific secondary antibodies, visualization, and quantification of the immunoblots was conducted as mentioned above.

Gelatin zymography

MMP-2 and MMP-9 enzymatic activity in the left brain hemispheres was measured as previously described (Soejima et al. 2013). Equal amounts of protein (50µg/sample) were diluted in zymography sample buffer and loaded onto gelatin zymogram gels (10%

polyacrylamide containing 0.1% gelatin; Invitrogen, Grand Island, NY). Electrophoresis was performed at 125V for 2 hours. Following that, zymogram gels were submerged into denature buffer (Bio-Rad) for 1 hour and incubated in development buffer for 48 hours. Staining was performed with 0.5% coomassie blue G-250 in 30% methanol and 10% acetic acid for 1 hour and gels were destained until visualization of clear bands on blue background. Human MMP-9 (Chemicon, Temecula, CA) was used as gelatinase standard. The gel image was taken and densitometrically quantified using the software ImageJ.

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean. Evans blue, Western blot, immunoprecipitation, and zymography measurements were normalized to the mean value of Sham. Fisher exact test was used in two group comparison for the statistical evaluation of mortality. Multiple comparisons were statistically evaluated using one-way analysis of variance (ANOVA) or Kruskal-Wallis ANOVA on Ranks followed by Tukey's post-hoc test. Statistical significance was considered at a p value of less than 0.05.

Results

Mortality and severity of experimental SAH

One hundred and forty-four male Sprague-Dawley rats were subjected to either sham operation ($n=33$) or SAH induction ($n=111$). Twenty SAH rats were excluded due to mild SAH grades (score <9). Fourteen animals died within 24 hours of SAH induction (overall mortality of 9.72%), 8 rats thereof from the Vehicle (16.67%) and 6 from the Imatinib-120mg group (13.95%). The mortality rate was not statistically different between treated and untreated SAH animals ($p>0.05$). The remaining 110 rats were subdivided into the following groups: Sham ($n=33$), Vehicle ($n=38$), Imatinib-40mg ($n=6$), and Imatinib-120mg ($n=33$). The injury severity for each time point was similar amongst all SAH groups ($p>0.05$; Fig.1).

Imatinib treatment preserved BBB integrity and improved neurological function at 24 hours following SAH induction

The PDGFR antagonist, imatinib (40mg/kg or 120mg/kg) was administered intraperitoneally at 1 hour following SAH induction. To assess BBB integrity, Evans blue was administered intravenously and post-mortem quantifications of extravasated dye were conducted in brain specimens. All rats subjected to SAH presented with significantly higher levels of extravasated dye than sham-operated animals ($p<0.05$; Fig. 2A). At 24 hours following SAH induction, higher levels of extravasated dye were detected in the left (ipsilateral) as compared to the right (contralateral) brain hemispheres. High dose treatment (Imatinib-120mg) significantly reduced the amount of extravasated dye in the left brain hemisphere; whereas high as well as low dose treatment (Imatinib-40mg) reduced the level of extravasated dye within the right brain hemisphere ($p<0.05$ compared to vehicle).

Brain edema (brain water content) was evaluated via the wet weight/dry weight method. At 24 hours after surgery, significant brain edema was found in SAH animals that received vehicle administration ($p<0.05$ compared to Sham; Fig. 2B). In contrast, treated rats

(Imatinib-120mg) demonstrated a brain water content similar to sham-operated animals ($p>0.05$). Moreover, high dose imatinib treatment (Imatinib-120mg) resulted in significant edema reduction within the left brain hemisphere ($p<0.05$ compared to Vehicle). No significant changes in right hemispheric brain edema were found after imatinib administration ($p>0.05$ compared to Vehicle). Brain water contents within the cerebellum and brain stem were similar between treated and untreated SAH animals ($p>0.05$; data not shown).

Sensorimotor neurological function was evaluated prior to euthanasia at 24 hours following SAH induction or sham operation. A deficit in sensorimotor neurological function was observed in treated and untreated SAH animals ($p<0.05$ compared to Sham; Fig. 2C); however, low dose (Imatinib-40mg) as well as high dose imatinib (Imatinib-120mg) significantly improved sensorimotor deficits compared to vehicle animals ($p<0.05$). No significant differences were observed between both treatment groups ($p>0.05$).

Body weights of all experimental animals were measured at 24 hours following SAH induction or sham surgery. In comparison to the baseline pre-operative weights, vehicle animals demonstrated a significant loss of body weight ($p<0.05$ compared to Sham; Fig. 2D). The degree of body weight loss was similar in the Sham and Imatinib-40mg group ($p>0.05$); however, SAH animals that received high dose treatment (Imatinib-120mg) demonstrated significantly less weight loss than rats in the Sham, Vehicle, and Imatinib-40mg groups ($p<0.05$).

Effects of imatinib treatment on brain edema, neurological deficits, and weight loss at 72 hours following SAH induction

Significant brain edema was measured in left and right brain hemispheres of SAH rats at 72 hours following hemorrhage induction ($p<0.05$ compared to Sham; Fig. 3A); however, no significant differences were observed between treated and untreated SAH animals ($p>0.05$). Similarly, sensorimotor deficits were significantly more severe in SAH animals as compared to sham-operated rats ($p<0.05$; Fig. 3B). High dose imatinib treatment (Imatinib-120mg) tendentially improved neurological function in SAH animals; however, without reaching statistical significance ($p=0.09$ compared to Vehicle; Fig. 3B). In comparison to the baseline pre-operative body weights, vehicle animals demonstrated a significant loss of body weight at 72 hours after surgery ($p<0.05$ compared to Sham; Fig. 3C). The extent of body weight loss was similar in the Sham and Imatinib-120mg group ($p>0.05$); and SAH animals that received high dose treatment (Imatinib-120mg) demonstrated significantly less weight loss than rats in the Vehicle group ($p<0.05$).

Effects of imatinib treatment on total as well as phosphorylated PDGFR, c-Src, JNK and c-Jun expressions at 24 hours following SAH induction

Western blot and immunoprecipitation analyses were used to quantify the expressions of total and phosphorylated PDGFR- α , c-Src, JNK, and c-Jun in the left (ipsilateral) brain hemispheres at 24 hours after surgery. No significant different PDGFR- α levels were measured in sham-operated and SAH animals ($p>0.05$; Fig. 4A). The ratio of phosphorylated and therefore activated to total receptor expression (p-PDGFR- α /PDGFR-

α), however, was increased in the Vehicle group ($p < 0.05$ compared to Sham, Fig. 4B). This increase was effectively reversed by imatinib treatment ($p < 0.05$; Fig. 4B).

Experimental SAH resulted in an increase in phosphorylated and therefore activated c-Src (p-c-Src), JNK (p-JNK) and c-Jun (p-c-Jun) with relatively constant levels of the respective total proteins (c-Src, JNK, and c-Jun; Fig. 5A). These changes resulted in a significant increase of the p-c-Src/c-Src ratio following SAH ($p < 0.05$ compared to Sham, Fig. 5B); however, imatinib treatment (Imatinib-120mg) significantly reduced the p-c-Src/c-Src ratio as compared to vehicle animals ($p < 0.05$). Similarly, SAH induction resulted in significantly increased p-JNK/JNK and p-c-Jun/c-Jun ratios ($p < 0.05$ compared to Sham; Fig. 5C and D). Imatinib treatment (Imatinib-120mg), however, resulted in a significant reduction of p-JNK/JNK as well as p-c-Jun/c-Jun ($p < 0.05$ compared to Vehicle) at 24 hours after SAH induction.

Imatinib treatment reduced MMP-9 activity at 24 hours following SAH induction

Gelatin zymography analysis was used to evaluate the enzymatic activities of MMP-9 and MMP-2 in the left (ipsilateral) brain hemispheres at 24 hours after SAH induction or sham surgery. MMP-9 as well as MMP-2 enzymatic activities were found significantly increased in treated and untreated SAH animals ($p < 0.05$ compared to Sham; Fig. 6). Imatinib treatment (Imatinib-120mg) significantly reduced the activity of MMP-9 in the left brain hemisphere of SAH animals at 24 hours after surgery ($p < 0.05$ compared to vehicle). MMP-2 activity was also found reduced in the treatment group; however, statistical significance was not achieved ($p = 0.08$ compared to Vehicle).

Discussion

This current study evaluated imatinib as a potential therapy for EBI following experimental SAH. High dose imatinib treatment (120mg/kg) reduced Evans blue extravasation into the brain; whereas low dose treatment (40mg/kg) reduced BBB disruption only in the less severely injured (right) brain hemisphere. For this reason, we used the high dose treatment for all subsequent experiments.

Evans blue extravasation assays are commonly utilized to measure the extent of BBB disruption in preclinical rodent models of ischemic, hemorrhagic, and traumatic brain injury. This technique implements a systemic administration of Evans blue dye, which has a high binding affinity for serum albumin (Hawkins and Egleton 2006; Reeve 1957). Evans blue-labeled albumin cannot readily cross the BBB under normal physiologic conditions. Therefore, spectrophotometric determination of Evans blue in the injured brain is a simple and reliable way to estimate permeability of the BBB to macromolecules (Manaenko et al. 2011). Several pathological factors are involved in BBB disintegration, including degradation of collagen within the basal lamina of cerebral vessels, decrease in tight and adherens junction proteins, physical widening of inter-epithelial spaces, as well as apoptosis of cells constituting the BBB (Gules et al. 2003; Sehba et al. 2004). Disruption of the BBB occurs as early as 10 minutes after SAH onset and peaks approximately 24 hours thereafter (Germano et al. 2000; Tso and Macdonald 2014). BBB breakdown facilitates the infiltration

of serum proteins into brain parenchyma, which amplifies inflammation, leading to further neuronal damage and edema formation (Petty and Lo 2002).

The degree of brain edema is a strong predictor for cognitive dysfunction, mortality and poor outcomes in SAH patients (Claassen et al. 2002). Brain edema can be classified into a vasogenic and cytotoxic subtype. Vasogenic brain edema defines an extracellular fluid accumulation as the direct consequence of BBB disruption, whereas cytotoxic brain edema refers to intracellular water accumulation due to failure of energy-dependent mechanism of ion extrusion (Nag et al. 2009). Both types of brain edema occur during SAH-induced formation of EBI (Nag et al. 2009; Yang et al. 1994). We evaluated the level of brain edema following experimental SAH via the wet weight/dry weight method, and found that imatinib treatment effectively reduced brain edema at 24 hours after SAH induction, which was paralleled by improved sensorimotor functions in treated animals. The applied method, however, does not differentiate between brain edema subtypes.

Body weight is a nonspecific indicator for an animal's overall condition (Duris et al. 2011b). In our experiments, vehicle animals demonstrated a significantly greater weight loss than sham-operated rats, which identifies the experimentally induced injury as a considerable stressor. Imatinib-treated animals; however, demonstrated improved body weights. In fact, no significant difference in weight loss was found between the Sham and the Imatinib-40mg group, whereas high dose imatinib treatment significantly reduced weight loss compared to all groups at 24 hours after surgery. These results suggest that imatinib administration improved the overall condition of rats subjected to SAH. Significant adverse effects of imatinib treatment may include water retention, liver toxicity, and bleeding. These side effects should be further assessed in rodents before starting Phase 1 clinical trials with relatively high doses, as used in our experiments (120 mg/kg).

Excessive PDGFR stimulation has been observed in SAH patients as well as in experimental studies of hemorrhagic stroke. Gaetani *et al.* found high levels of the receptor's endogenous ligand (platelet derived growth factor (PDGF)) in cerebrospinal fluid specimens of SAH patients, early after symptom onset (Gaetani et al. 1997). Proto-oncogene c-Src has been demonstrated to transactivate other protein tyrosine kinases, including PDGFRs (Tsai et al. 2014), and increased levels of c-Src were found in cerebral arteries of dogs subjected to experimental SAH (Kusaka et al. 2003). Moreover, binding of TNF- α to its cytokine receptor facilitates c-Src activation; and proinflammatory cytokines (such as TNF- α) play an important role in the development of EBI after SAH (Fujii et al. 2013; Tsai et al. 2014). Molecular interactions between PDGFR and c-Src lead to receptor activation as well as to c-Src autophosphorylation at Tyr419, which further promotes its kinase activity (Amanchy et al. 2009). Our results confirmed increased expressions of activated PDGFR- α and phosphorylated c-Src (Tyr419) in the brain of SAH animals. Based on these findings, and in agreement with previous literature, we assume that PDGFR- α activation, following experimental SAH, occurred directly via PDGF binding as well as indirectly via c-Src-induced receptor transactivation (Fig. 6B).

Mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal kinases (JNKs) are critical downstream targets of PDGFR signaling (Ma et al. 2011). JNK activation, as a

consequence of hemorrhagic stroke, has been associated with BBB disruption due to a reduced expression of barrier-forming tight junction proteins such as claudin-5 and ZO-1 (Chen et al. 2012; Ma et al. 2011). Pharmacological inhibition of JNK, however, restored tight junction proteins and attenuated brain edema after SAH (Chen et al. 2012). Activated JNK is phosphorylated on specific threonine (Thr183) and tyrosine (Tyr185) residues. In its phosphorylated form, JNK can activate c-Jun (via phosphorylation on Ser63/73), which then translocates into the nucleus to constitute the AP-1 transcription factor (Loesch et al. 2010). AP-1 is composed of homodimers of the Jun family or heterodimers of c-Jun combined with another transcription factor such as c-Fos (Tsai et al. 2014). Activated c-Jun/AP-1 leads to cellular responses through integrated gene transcription, including MMP-9 gene transcription (Loesch et al. 2010; Tsai et al. 2014). Brilliant blue G, a selective P2X7 purinergic receptor antagonist, has been shown to reduce sensorimotor deficits as well as brain edema at 24 and 72 hours after induction of experimental SAH in rats (Chen et al. 2013). In accordance with our current findings, the protective effects of brilliant blue G were also associated with molecular inhibition of cerebral MAPK and its downstream targets.

The detrimental role of MMPs in the progression of ischemic and hemorrhagic brain injury has been well recognized (Dong et al. 2009; Ma et al. 2011). Especially the gelatinases MMP-2 and MMP-9 were found up-regulated after hemorrhagic stroke (Ma et al. 2011). MMP-2 and MMP-9 degrade extracellular matrix proteins such as type IV collagen of cerebral microvessels, resulting in increased BBB leakage or even vessel rupture. MMP-9 gene knockout, or administration of synthetic MMP inhibitors, effectively preserved BBB integrity and reduced the formation of vasogenic brain edema after experimental stroke (Asahi et al. 2000; Dong et al. 2009). We found higher levels of activated JNK and c-Jun as well as increased enzymatic activities of MMP-2 and MMP-9 in the brain of SAH rats. Imatinib-induced inhibition of PDGFR signaling, however, reversed these molecular changes, but did not significantly reduce MMP-2 activity. In a previous study, experimental SAH was induced via injection of autologous blood into the rat's prechiasmatic cistern, and administration of the MMP-9 inhibitor, SB-3CT, reduced the number of apoptotic neurons within the cerebral cortex. (Guo et al. 2011). Furthermore, osteopontin treatment prevented significant weight loss and reduced sensorimotor deficits, BBB disruption, and brain edema formation in rats subjected to experimental SAH (Suzuki et al. 2010). These effects were also associated with inhibition of MMP-9 and consequent preservation of cerebral microvessels.

Imatinib is an anti-cancer drug approved for the treatment of chronic myelogenous leukemia and other malignancies (Peggs and Mackinnon 2003). It inhibits several tyrosine kinases, including PDGFR- α , and has been shown to reduce brain edema following ischemic stroke as well as following initiation of plasminogen activator therapy (Su et al. 2008). Furthermore, imatinib-associated inhibition of PDGFR- α preserved BBB integrity in a mouse model of intracerebral hemorrhage (Ma et al. 2011). PDGFR activation is implicated in the pathogenesis of CVS following SAH, and imatinib treatment prevented the occurrence of CVS at least partially through normalization of the tenascin-C expression (Shiba et al. 2014; Shiba et al. 2012). Moreover, imatinib treatment resulted in a reduced migration of immune cells, specifically leukocytes, through the BBB of rats subjected to myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis

(Adzemovic et al. 2013). Indeed, a broad reduction of cytokines and their receptors was demonstrated in imatinib-treated animals; thus, the observed BBB-protective effects were possibly amplified by this treatment's anti-inflammatory properties.

Our current study has several limitations. First, we did not implement a multiple treatment regime for the evaluation of brain edema and neurological deficits at 72 hours after SAH induction, which we believe would have improved the outcome at this time point. Second, we did not quantify the expression of activated PDGFR- β , as PDGFR- α has been shown to play a more important role in the development of hemorrhage-induced brain edema formation. Third, BBB permeability and the extent of brain edema following SAH can be evaluated with more innovative methodology such as magnetic resonance imaging; however, this study implemented post-mortem quantifications of Evans blue extravasation and brain water content.

In conclusion, PDGFR- α signaling may contribute to BBB disruption and consequent edema formation via JNK/c-Jun-mediated MMP-9 activation following experimental SAH; however, PDGFR- α inhibition via imatinib may preserve the BBB integrity. To our best knowledge, this is the first study evaluating the effects of imatinib treatment on SAH-induced BBB disruption and brain edema formation. Although protective mechanisms remain to be further elucidated, we believe that targeting PDGFR- α signaling may be an advantageous strategy to ameliorate EBI following SAH.

Acknowledgments

Financial support for this current study was provided by the following grant: P01NS082184 to Drs. J.H. Zhang and J. Tang.

References

- Amanchy R, Zhong J, Hong R, Kim JH, Gucek M, Cole RN, Molina H, Pandey A. Identification of c-Src tyrosine kinase substrates in platelet-derived growth factor receptor signaling. *Mol Oncol*. 2009; 3(5-6):439–450. [PubMed: 19632164]
- Asahi M, Asahi K, Jung JC, del Zoppo GJ, Fini ME, Lo EH. Role for matrix metalloproteinase 9 after focal cerebral ischemia: effects of gene knockout and enzyme inhibition with BB-94. *J Cereb Blood Flow Metab*. 2000; 20(12):1681–1689. [PubMed: 11129784]
- Chen D, Wei XT, Guan JH, Yuan JW, Peng YT, Song L, Liu YH. Inhibition of c-Jun N-terminal kinase prevents blood-brain barrier disruption and normalizes the expression of tight junction proteins claudin-5 and ZO-1 in a rat model of subarachnoid hemorrhage. *Acta Neurochir (Wien)*. 2012; 154(8):1469–1476. discussion 1476. [PubMed: 22661329]
- Claassen J, Carhuapoma JR, Kreiter KT, Du EY, Connolly ES, Mayer SA. Global cerebral edema after subarachnoid hemorrhage: frequency, predictors, and impact on outcome. *Stroke*. 2002; 33(5): 1225–1232. [PubMed: 11988595]
- Dong X, Song YN, Liu WG, Guo XL. Mmp-9, a potential target for cerebral ischemic treatment. *Curr Neuropharmacol*. 2009; 7(4):269–275. [PubMed: 20514206]
- Duris K, Manaenko A, Suzuki H, Rolland W, Tang J, Zhang JH. Sampling of CSF via the Cisterna Magna and Blood Collection via the Heart Affects Brain Water Content in a Rat SAH Model. *Translational stroke research*. 2011a; 2(2):232–237. [PubMed: 21666823]
- Duris K, Manaenko A, Suzuki H, Rolland WB, Krafft PR, Zhang JH. $\alpha 7$ nicotinic acetylcholine receptor agonist PNU-282987 attenuates early brain injury in a perforation model of subarachnoid hemorrhage in rats. *Stroke*. 2011b; 42(12):3530–3536. [PubMed: 21960575]

- Fujii M, Yan J, Rolland WB, Soejima Y, Caner B, Zhang JH. Early brain injury, an evolving frontier in subarachnoid hemorrhage research. *Transl Stroke Res*. 2013; 4(4):432–446. [PubMed: 23894255]
- Gaetani P, Tancioni F, Grignani G, Tartara F, Merlo EM, Brocchieri A, Rodriguez y Baena R. Platelet derived growth factor and subarachnoid haemorrhage: a study on cisternal cerebrospinal fluid. *Acta Neurochir (Wien)*. 1997; 139(4):319–324. [PubMed: 9202771]
- Garcia JH, Wagner S, Liu KF, Hu XJ. Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation. *Stroke*. 1995; 26(4):627–634. discussion 635. [PubMed: 7709410]
- Germano A, d'Avella D, Imperatore C, Caruso G, Tomasello F. Time-course of blood-brain barrier permeability changes after experimental subarachnoid haemorrhage. *Acta Neurochir (Wien)*. 2000; 142(5):575–580. discussion 580–571. [PubMed: 10898366]
- Gules I, Satoh M, Nanda A, Zhang JH. Apoptosis, blood-brain barrier, and subarachnoid hemorrhage. *Acta Neurochir Suppl*. 2003; 86:483–487. [PubMed: 14753491]
- Hawkins BT, Egleton RD. Fluorescence imaging of blood-brain barrier disruption. *J Neurosci Methods*. 2006; 151(2):262–267. [PubMed: 16181683]
- Johnston SC, Selvin S, Gress DR. The burden, trends, and demographics of mortality from subarachnoid hemorrhage. *Neurology*. 1998; 50(5):1413–1418. [PubMed: 9595997]
- Kreiter KT, Copeland D, Bernardini GL, Bates JE, Peery S, Claassen J, Du YE, Stern Y, Connolly ES, Mayer SA. Predictors of cognitive dysfunction after subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation*. 2002; 33(1):200–208.
- Kusaka G, Kimura H, Kusaka I, Perkins E, Nanda A, Zhang JH. Contribution of Src tyrosine kinase to cerebral vasospasm after subarachnoid hemorrhage. *J Neurosurg*. 2003; 99(2):383–390. [PubMed: 12924714]
- Linn FH, Rinkel GJ, Algra A, van Gijn J. Incidence of subarachnoid hemorrhage: role of region, year, and rate of computed tomography: a meta-analysis. *Stroke*. 1996; 27(4):625–629. [PubMed: 8614919]
- Loesch M, Zhi HY, Hou SW, Qi XM, Li RS, Basir Z, Iftner T, Cuenda A, Chen G. p38gamma MAPK cooperates with c-Jun in trans-activating matrix metalloproteinase 9. *J Biol Chem*. 2010; 285(20):15149–15158. [PubMed: 20231272]
- Ma Q, Huang B, Khatibi N, Rolland W 2nd, Suzuki H, Zhang JH, Tang J. PDGFR-alpha inhibition preserves blood-brain barrier after intracerebral hemorrhage. *Ann Neurol*. 2011; 70(6):920–931. [PubMed: 22190365]
- Macdonald RL, Higashida RT, Keller E, Mayer SA, Molyneux A, Raabe A, Vajkoczy P, Wanke I, Bach D, Frey A, Marr A, Roux S, Kassell N. Clazosentan, an endothelin receptor antagonist, in patients with aneurysmal subarachnoid haemorrhage undergoing surgical clipping: a randomised, double-blind, placebo-controlled phase 3 trial (CONSCIOUS-2). *Lancet Neurol*. 2011; 10(7):618–625. [PubMed: 21640651]
- Macdonald RL, Kassell NF, Mayer S, Ruefenacht D, Schmiedek P, Weidauer S, Frey A, Roux S, Pasqualin A. Clazosentan to overcome neurological ischemia and infarction occurring after subarachnoid hemorrhage (CONSCIOUS-1): randomized, double-blind, placebo-controlled phase 2 dose-finding trial. *Stroke*. 2008; 39(11):3015–3021. [PubMed: 18688013]
- Manaenko A, Chen H, Kammer J, Zhang JH, Tang J. Comparison Evans Blue injection routes: Intravenous versus intraperitoneal, for measurement of blood-brain barrier in a mice hemorrhage model. *J Neurosci Methods*. 2011; 195(2):206–210. [PubMed: 21168441]
- Nag S, Manias JL, Stewart DJ. Pathology and new players in the pathogenesis of brain edema. *Acta Neuropathol*. 2009; 118(2):197–217. [PubMed: 19404652]
- Ostrowski RP, Colohan AR, Zhang JH. Molecular mechanisms of early brain injury after subarachnoid hemorrhage. *Neurol Res*. 2006; 28(4):399–414. [PubMed: 16759443]
- Peggs K, Mackinnon S. Imatinib mesylate--the new gold standard for treatment of chronic myeloid leukemia. *N Engl J Med*. 2003; 348(11):1048–1050. [PubMed: 12637616]
- Petty MA, Lo EH. Junctional complexes of the blood-brain barrier: permeability changes in neuroinflammation. *Progress in neurobiology*. 2002; 68(5):311–323. [PubMed: 12531232]
- Reeve EB. The contribution of I 131-labeled proteins to measurements of blood volume. *Ann N Y Acad Sci*. 1957; 70(1):137–147. [PubMed: 13488261]

- Schwartz AY, Masago A, Sehba FA, Bederson JB. Experimental models of subarachnoid hemorrhage in the rat: a refinement of the endovascular filament model. *J Neurosci Methods*. 2000; 96(2):161–167. [PubMed: 10720681]
- Sehba FA, Mostafa G, Knopman J, Friedrich V Jr, Bederson JB. Acute alterations in microvascular basal lamina after subarachnoid hemorrhage. *J Neurosurg*. 2004; 101(4):633–640. [PubMed: 15481718]
- Shiba M, Fujimoto M, Imanaka-Yoshida K, Yoshida T, Taki W, Suzuki H. Tenascin-C Causes Neuronal Apoptosis After Subarachnoid Hemorrhage in Rats. *Transl Stroke Res*. 2014
- Shiba M, Suzuki H, Fujimoto M, Shimajo N, Imanaka-Yoshida K, Yoshida T, Kanamaru K, Matsushima S, Taki W. Imatinib mesylate prevents cerebral vasospasm after subarachnoid hemorrhage via inhibiting tenascin-C expression in rats. *Neurobiol Dis*. 2012; 46(1):172–179. [PubMed: 22300707]
- Soejima Y, Hu Q, Krafft PR, Fujii M, Tang J, Zhang JH. Hyperbaric oxygen preconditioning attenuates hyperglycemia-enhanced hemorrhagic transformation by inhibiting matrix metalloproteinases in focal cerebral ischemia in rats. *Exp Neurol*. 2013
- Su EJ, Fredriksson L, Geyer M, Folestad E, Cale J, Andrae J, Gao Y, Pietras K, Mann K, Yepes M, Strickland DK, Betsholtz C, Eriksson U, Lawrence DA. Activation of PDGF-CC by tissue plasminogen activator impairs blood-brain barrier integrity during ischemic stroke. *Nat Med*. 2008; 14(7):731–737. [PubMed: 18568034]
- Suarez JJ, Tarr RW, Selman WR. Aneurysmal subarachnoid hemorrhage. *N Engl J Med*. 2006; 354(4):387–396. [PubMed: 16436770]
- Sugawara T, Ayer R, Jadhav V, Zhang JH. A new grading system evaluating bleeding scale in filament perforation subarachnoid hemorrhage rat model. *J Neurosci Methods*. 2008; 167(2):327–334. [PubMed: 17870179]
- Suzuki H, Ayer R, Sugawara T, Chen W, Sozen T, Hasegawa Y, Kanamaru K, Zhang JH. Protective effects of recombinant osteopontin on early brain injury after subarachnoid hemorrhage in rats. *Crit Care Med*. 2010; 38(2):612–618. [PubMed: 19851092]
- Tsai CL, Chen WC, Lee IT, Chi PL, Cheng SE, Yang CM. c-Src-dependent transactivation of PDGFR contributes to TNF-alpha-induced MMP-9 expression and functional impairment in osteoblasts. *Bone*. 2014; 60C:186–197. [PubMed: 24361597]
- Tso MK, Macdonald RL. Subarachnoid Hemorrhage: a Review of Experimental Studies on the Microcirculation and the Neurovascular Unit. *Transl Stroke Res*. 2014
- Uyama O, Okamura N, Yanase M, Narita M, Kawabata K, Sugita M. Quantitative evaluation of vascular permeability in the gerbil brain after transient ischemia using Evans blue fluorescence. *J Cereb Blood Flow Metab*. 1988; 8(2):282–284. [PubMed: 3343300]
- Wilkins RH. Cerebral vasospasm. *Crit Rev Neurobiol*. 1990; 6(1):51–77. [PubMed: 2225095]
- Yang GY, Betz AL, Chenevert TL, Brunberg JA, Hoff JT. Experimental intracerebral hemorrhage: relationship between brain edema, blood flow, and blood-brain barrier permeability in rats. *J Neurosurg*. 1994; 81(1):93–102. [PubMed: 8207532]

A **B** **Figure 1.**

Representative photographs showing SAH grading of increasing severity (**A**) and statistical analysis of SAH grading at 24 and 72 hours (**B**) after surgery. Data are expressed as mean $\pm$ SEM. NS=not significant. Experimental animals used for the assessment of SAH severity at 24 hours were subdivided into the following groups: Vehicle (n=32), Imatinib-40mg (n=6), Imatinib-120mg (n=27); and for the 72 hour time point: Vehicle (n=6) and Imatinib-120mg (n=6)

Figure 2.

Statistical analysis of Evans blue extravasation (A), brain edema (B), neurological function (C), and loss of body weight (D) at 24 hours after SAH induction or sham surgery. Data are expressed as mean $\pm$ SEM. * $p < 0.05$ compared to Sham, # $p < 0.05$ compared to Vehicle, † $p < 0.05$ compared to Imatinib-120mg. Experimental animals used for the assessment of Evans blue extravasation were subdivided into the following groups: Sham (n=8), Vehicle (n=10), Imatinib-40mg (n=6), and Imatinib-120mg (n=7). Experimental animals used for the assessment of brain edema were subdivided into the following groups: Sham (n=7), Vehicle (n=10), and Imatinib-120mg (n=8). Neurological functions and loss of body weight at 24

hours after surgery were evaluated in animals allocated for the Evans blue and brain edema experiments.

Figure 3.

Statistical analysis of brain edema (**A**), neurological function (**B**), and loss of body weight (**C**) at 72 hours after SAH induction or sham surgery. Data are expressed as mean $\pm$ SEM. * $p < 0.05$ compared to Sham, # $p < 0.05$ compared to Vehicle. Experimental animals used for these assessments were subdivided into the following groups: Sham, Vehicle, and Imatinib-120mg. N=6 in each group.

Figure 4.

Representative immunoblots and densitometric quantification of PDGFR-α/β-Actin (**A**) and p-PDGFR-α/PDGFR-α (**B**) levels in the left brain hemisphere at 24 hours after SAH induction or sham surgery. Data are expressed as mean ± SEM. * $p < 0.05$ compared to Sham, # $p < 0.05$ compared to Vehicle. Experimental animals used for this assessment were subdivided into the following groups: Sham, Vehicle, and Imatinib-120mg. N=6 in each group.

Figure 5.

Representative Western blots (**A**) and densitometric quantification of p-c-Src/c-Src/ β -Actin (**B**), p-JNK/JNK/ β -Actin (**C**), and p-c-Jun/c-Jun/ β -Actin (**D**) levels in the left brain hemisphere at 24 hours after SAH induction or sham surgery. Data are expressed as mean $\pm$ SEM. * $p < 0.05$ compared to Sham, # $p < 0.05$ compared to Vehicle. Experimental animals used for this assessment were subdivided into the following groups: Sham, Vehicle, and Imatinib-120mg. N=6 in each group.

Figure 6.

Gelatin zymography assay and densitometric quantification of MMP-9 and MMP-2 activity in the left brain hemisphere at 24 hours after SAH induction or sham surgery (A). Data are expressed as mean $\pm$ SEM. * $p < 0.05$ compared to Sham, # $p < 0.05$ compared to Vehicle. Experimental animals used for this assessment were subdivided into the following groups: Sham, Vehicle, and Imatinib-120mg. N=6 in each group. Proposed mechanism of PDGFR- α induced BBB breakdown via c-Src/JNK/c-Jun and MMP-9 following SAH (B).