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Effects of crenolanib, a non-selective inhibitor of PDGFR, in a mouse model of transient middle cerebral artery occlusion

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Abstract

Neurogenesis in the subventricular zone (SVZ) plays a vital role in neurologic recovery after stroke. However, only a small fraction of newly generated neuroblasts from the SVZ will survive long-term. Successful migration and survival of neuroblasts requires angiogenesis, lesion-derived chemo-attractants, and appropriate local microenvironments, which are partly regulated by the platelet-derived growth factor receptor (PDGFR) signaling pathway. In this study, we investigated the effects of PDGFR inhibition in a mouse model of transient middle cerebral artery occlusion (MCAO). We blocked the pathway using a selective pan-PDGFR inhibitor, crenolanib, during the acute post-MCAO phase (days 1–3) or during the sub-acute phase (days 7–9). Downregulating the PDGFR signaling pathway with crenolanib from day 1 to day 3 after MCAO significantly decreased the migration of neuroblasts from the SVZ to the peri-infarct region, decreased angiogenesis, and lowered expression of vascular endothelial growth factor, stromal cell-derived factor-1, and monocyte chemotactic protein-1. Downregulation of the PDGFR signaling pathway

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on days 7 to 9 with crenolanib significantly increased apoptosis of the neuroblasts that had migrated to the peri-infarct region, increased the number of activated microglia, and decreased the expression of brain-derived neurotrophic factor, neurotrophin-3, and interleukin-10. Crenolanib treatment increased the apoptosis of pericytes and decreased the pericyte/vascular coverage, but had no effects on apoptosis of astrocytes. We conclude that PDGFR signaling pathway plays a vital role in the SVZ neurogenesis after stroke, it can also affect angiogenesis, lesion-derived chemo-attractants and local microenvironment, which are of importance in the stroke-induced neurogenesis.

Keywords

neuroblasts; neurogenesis; PDGFR; stroke; subventricular zone

INTRODUCTION

Neurogenesis is a promising therapeutic target for ischemic stroke. Researchers have shown that newly generated neuroblasts from the subventricular zone (SVZ) can migrate into the infarct area and promote neurologic recovery after stroke by differentiating into mature neurons and modulating local immunoreactions (Parent et al., 2002, Tobin et al., 2014). Neurogenesis has been widely regarded as a prognostic indicator in stroke research, and its promotion has been shown to be neuroprotective (Bravo-Ferrer et al., 2017, Song et al., 2017). Conversely, inhibition of SVZ neurogenesis can aggravate neurologic damage and worsen stroke outcomes (Wang et al., 2017). The major problem with endogenous neurogenesis as a potential mechanism for stroke recovery is that only a small fraction of the new neurons survive long-term (Ekdahl et al., 2009). Although neurogenesis is activated in the SVZ after stroke, more than 80% of newly generated neuroblasts die within 28 days (Arvidsson et al., 2002). Protecting the migration and survival of SVZ neuroblasts after stroke may enhance the efficiency of endogenous neurogenesis and provide a potential therapeutic method for stroke treatment.

Platelet-derived growth factors (PDGFs) and their receptors, namely PDGFR α and PDGFR β , are widely expressed in the central nervous system. The PDGF receptor (PDGFR) signaling pathway participates in the embryonic development of mammal brain and can regulate pathophysiologic processes in many neurologic diseases. Studies have indicated that activation of the PDGFR α signaling pathway at the acute stage of stroke is associated with impaired cerebrovascular permeability (Su et al., 2008) but that activation of the PDGFR β signaling pathway has a positive effect on brain repair after cerebral ischemia. PDGFR β -deficient mice exhibited more vascular leakage, greater infarction volume, and slower recovery of behavioral function after middle cerebral artery occlusion (MCAO) than did their control counterparts (Shen et al., 2012). These results indicate that the PDGFR signaling pathway is a potential therapeutic target for ischemic stroke. However, the underlying mechanisms by which the PDGFR signaling pathway influences migration and survival of neuroblasts after ischemic stroke remains unknown.

PDGFR signaling regulates the function of the neurovascular unit, which consists of vasculature, neurons, astrocytes, microglia, and pericytes. The neurovascular unit provides the newly generated vessels (Font et al., 2010), chemo-attractants such as stromal cell-derived factor-1 (SDF-1) and monocyte chemotactic protein 1 (MCP-1), and microenvironments (Arai et al., 2011) that are essential for the migration and survival of neuroblasts from the SVZ after stroke. Within the neurovascular unit, PDGFR α is expressed mainly on perivascular astrocytes, and PDGFR β is expressed mainly on pericytes and neural stem cells. Both astrocytes and pericytes are crucial for the secretion of lesion-derived chemo-attractants and regulation of local angiogenesis/microenvironments after ischemic stroke, but the role of the PDGFR signaling pathway in the migration and survival of neuroblasts remains unclear. In this study, by using the pan-PDGFR inhibitor crenolanib, we investigated the role of PDGFR signaling pathway in the migration and survival of SVZ neuroblasts, angiogenesis, lesion-derived chemo-attractants, local microenvironment and BBB permeability after acute ischemic stroke.

EXPERIMENTAL PROCEDURES

Animals and ethics statement

In total, 213 male C57BL/6 mice (25–30 g, 12–14 weeks old) were purchased from the Animal Experimental Center of Zhengzhou University and housed in plastic cages with free access to food and water. The animal room was maintained on a 12-h light/dark cycle at a constant temperature of $22\pm 1^{\circ}\text{C}$. The study was carried out in accordance with the recommendations of the Guidelines on the Care and Use of Animals for Scientific Purpose (National Advisory Committee for Laboratory Animal Research), and the protocol was approved by the Animal Care and Use Committee of the Fifth Affiliated Hospital of Zhengzhou University. We made all efforts to minimize the number of animals and their suffering.

Transient MCAO

We subjected mice to the transient MCAO model using the intraluminal filament technique, as previously described (Wang et al., 2015b). A 6.0 monofilament nylon suture with silicone-coated tip was introduced into the origin of the middle cerebral artery and left in place for 45 min until reperfusion. We defined a successful MCAO as a decrease in cerebral blood flow of more than 80% as measured by laser Doppler flowmetry (Moor Instruments, Devon, UK). For the sham operation, mice underwent the same procedure except that the filaments were inserted into the artery opening and then withdrawn immediately.

Treatment and groups

We used crenolanib to study the role of PDGFR signaling in the migration and survival of neuroblasts from the SVZ in MCAO mice. Crenolanib is a potent and selective inhibitor of PDGFR α/β with K_d of 2.1 nM/3.2 nM. It has been used previously for study of the PDGFR signaling pathway in C57BL/6 mouse disease models (He et al., 2015, Makino et al., 2017). We randomly assigned mice to four groups: sham-operated mice treated with vehicle (Sham + vehicle), sham-operated mice treated with crenolanib (15 mg/kg, dissolved in 30% PEG-400+0.5% Tween-80+5% propylene glycol; Sham+crenolanib), MCAO mice treated

with vehicle (MCAO+vehicle), and MCAO mice treated with crenolanib (MCAO +crenolanib). The MCAO+crenolanib group was divided into two subgroups: one was treated with crenolanib on days 1–3 and/or with 5-bromo-2'-deoxyuridine (BrdU, 50 mg/kg, Sigma-Aldrich, St Louis, MO, USA) (Wang et al., 2013) (Li et al., 2016) on days 1–7 after MCAO, and the second was treated with crenolanib on days 7–9 after MCAO. Vehicle, crenolanib, and BrdU were all administered into mice via intraperitoneal injection.

Neurologic function assessment

We used the modified neurologic severity score (mNSS) to test the neurologic function of mice from different groups as previously described (Wang et al., 2015a). The mNSS comprises tests on aspects of motor function, sensory function, balance, reflex, and general movement on a scale of 0–22 (0 = no deficit, and 22 = maximal deficit). Ten mice of each group were used for the mNSS test. The assessment was performed by an investigator blinded to groups on day 1 and then once a week until week 5 after the MCAO procedure.

Immunohistologic analysis

Eight mice from each group were used for immunohistologic analysis as previously described (Wu et al., 2015, Wang et al., 2017). Briefly, we deeply anesthetized mice with an overdose of chloral hydrate and perfused them transcardially with 0.01 mol/L phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.01 mol/L PBS (pH 7.4). Based on our established protocol (Zhao et al., 2015, Han et al., 2016, Wang et al., 2017), brain sections (20 μ m) were processed with Nissl staining to quantify infarct volume or stained with antibodies against PDGFR β (1:300, Abcam, Cambridge, MA, USA), GFAP (1:300, Santa Cruz Biotechnology, Dallas, TX, USA), doublecortin (DCX; 1:250, Santa Cruz Biotechnology), cleaved-caspase 3 (cCasp3; 1:500, Millipore, Billerica, MA, USA), CD31 (1:100, Abcam), BrdU (1:250, Abcam), Lectin fluorescein lycopersicon esculentum (tomato) (Lectin, 1:1000; Vector Laboratories, Burlingame, CA), SDF-1 (1:50, Proteintech, Sanying Biotechnology, Wuhan, China), MCP-1 (Proteintech), CD68 (1:500, Santa Cruz Biotechnology), occludin (1:250, Proteintech), and albumin (1:500, Santa Cruz Biotechnology). The infarct volume percentage was calculated by the equation: total infarct volume/contralateral hemispheric volume $\times$ 100%. For each section that contained infarction, we randomly chose three 10 $\times$, 20 $\times$ or 40 $\times$ fields in the peri-infarct areas to quantify DCX/PDGFR β -positive cells, /DCX/cCasp3-positive cells, CD31/BrdU-positive vessels, CD68-positive cells, PDGFR β /cCasp3-positive cells, PDGFR β -positive cells, Lectin-positive vessels, and GFAP/cCasp3/DAPI-positive cells. For each section that contained SVZ, we randomly chose three 10 $\times$ fields in areas between SVZ and infarction to quantify DCX-positive cells. All sections were observed and quantified under a fluorescence microscope (ZEISS Scope A1, ZEISS, Germany) by investigators blinded to the treatments and groups (Cheng et al., 2016).

ELISA analysis

Eight mice from each group were used to detect changes in vascular endothelial growth factor (VEGF), SDF-1, MCP-1, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) or interleukin (IL)-10 level in the ischemic border according to the manufacturer's protocol (NeoBioscience, Shenzhen, China, or Boster, Wuhan, China) (Lan et al., 2017).

Western blot analysis

Eight mice each from the MCAO+vehicle (1–3 d) and MCAO+crenolanib (1–3 d) groups were sacrificed for Western blot analysis of occludin and albumin levels in the peri-infarct area on day 3 after MCAO as previously described (Wang et al., 2015a). Protein samples from each group were separated on 8% glycine gel and transferred onto polyvinylidenedifluoride membranes. The membranes were blocked in 5% nonfat milk in PBS with 0.25% Tween-20 and stained with antibodies against occludin (1:250, Proteintech), albumin (1:500, Santa Cruz Biotechnology), or glyceraldehyde 3-phosphate dehydrogenase (1:2000, HangZhou Goodhere Biotechnology, Zhejiang, China) for 1 h at room temperature. After three washes in PBS containing Tween-20, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized by enhanced chemiluminescence detection kit (Cwbiotech, Beijing, China). An investigator blinded to the group quantified the optical density of the protein bands using Gel Analysis V 2.02 software (Clinx Science Instruments).

Statistical analysis

Statistical analysis was carried out with SPSS version 13.0. Results are expressed as mean $\pm$ SD. We used Fisher's exact test to examine differences in mortality rate; one-way ANOVA followed by the Bonferroni test or t-test to analyze histologic, ELISA, and Western blot differences; and repeated measures ANOVA followed by the Bonferroni test to determine changes in mNSS between groups. $p < 0.05$ was considered statistically significant.

RESULTS

Mortality rates

Twenty-five of 213 mice died during the experiment. The mortality rates were 0/32 (0%) in the sham+vehicle group, 0/32 (0%) in the sham+crenolanib group, 6/48 (12.5%) in the MCAO+vehicle (1–3 d) group, 9/51 (17.65%) in the MCAO+crenolanib (1–3 d) group, 2/18 (11.1%) in the MCAO+vehicle (7–9 d) group, and 8/32 (25%) in the MCAO+crenolanib (7–9 d) group. Mortality rates did not differ significantly among the four MCAO groups ($p > 0.05$).

Migration and survival of neuroblasts from the SVZ

Immunofluorescence analysis indicated that MCAO induced migration of DCX-positive neuroblasts from the SVZ to the infarction within 7 days after surgery (Fig. 1A–B). On day 7 post-surgery, fewer migrating neuroblasts were observed in the area between the SVZ and infarction in MCAO mice treated with crenolanib on days 1–3 than in MCAO mice treated with vehicle (Fig. 1A–B). We found majority of neuroblasts within SVZ or in peri-infarction area do not express PDGFR β (Fig. 1C–D). MCAO mice treated with crenolanib on days 7–9 after surgery also exhibited more cCasp-3-positive neuroblasts in the peri-infarct area on day 14 than did the MCAO+vehicle group (Fig. E–G). DCX-positive neuroblasts did not migrate out of the SVZ in sham groups, with or without crenolanib treatment (data not shown). mNSS testing showed that crenolanib treatment at the early stage of stroke (days 1 to 3), but

not at the sub-acute stage (days 7 to 9), significantly slowed neurologic recovery of MCAO mice (Fig. 1H).

Effect of crenolanib on angiogenesis and chemoattractants

MCAO mice treated with crenolanib (days 1–3) had fewer proliferative vessels (CD31/BrdU-positive vessels) in the peri-infarct area than did mice in the MCAO+vehicle group on day 7 post-surgery (Fig. 2A–D). ELISA analysis indicated that crenolanib treatment decreased the average level of VEGF in the ischemic border of MCAO mice, but no statistical difference was found (Fig. 2E). Immunofluorescence analysis and ELISA showed that expression of SDF-1 and MCP-1 in the ischemic border was less in the MCAO +crenolanib group than in the MCAO+vehicle group on day 7 after surgery (Fig. 2F–I). No differences in CD31/BrdU-positive vessels or levels of VEGF, SDF-1, and MCP-1 were found among the two sham groups (Fig. 2A–G).

Effect of crenolanib on inflammatory factors

Immunofluorescence analysis indicated that MCAO mice treated with crenolanib from day 7 to day 9 had more activated microglia (CD68-positive) in the peri-infarct region than did MCAO+vehicle mice on day 14 after surgery (Fig. 3A–B). ELISA analysis showed that crenolanib treatment also significantly decreased the levels of BDNF, NT-3, and IL-10 in the peri-infarct region on day 10 after MCAO (Fig. 3C–E). Crenolanib treatment did not affect CD68-positive cells or levels of BDNF, NT-3, and IL-10 in sham-treated mice (Fig. 3A–E).

Inhibition of PDGFR signaling with crenolanib decreases pericyte function

MCAO mice treated with crenolanib, either at the acute stage (days 1 to 3) or the subacute stage (days 7 to 9), had significantly more apoptotic pericytes (PDGFR β /cCasp3-positive cells) and a lower ratio of pericytes/vessels (number of PDGFR β cells/number of Lectin-positive vessels) in the peri-infarct area than did the corresponding control groups (Fig. 4A–B, D–E). Apoptosis of astrocytes in the peri-infarct region was unaffected by crenolanib treatment at either stage (Fig. 4C, F). Vehicle- and crenolanib-treated mice showed similar numbers of PDGFR β /cCasp3-positive cells, ratios of pericytes/vessels, and apoptotic astrocytes in the cortex (data not shown).

Inhibition of PDGFR signaling with crenolanib increases blood-brain barrier (BBB) permeability

Infarct volume was similar in MCAO mice treated with crenolanib on days 1–3 and those treated with vehicle (Fig. 5A and B). However, immunofluorescence and Western blot analysis indicated that blocking the PDGFR signaling pathway with crenolanib significantly increased BBB permeability on day 3 in MCAO mice (Fig. 4C–G).

DISCUSSION

Neurogenesis is necessary for recovery after stroke. We and others have shown that inhibiting the neurogenesis process deepens brain damage, and that increasing the proliferation of neural stem cells in the SVZ can promote better neurologic outcome after stroke (Song et al., 2017, Wang et al., 2017). In this study, we showed that the PDGFR

signaling pathway plays a vital role in post-stroke migration and survival of neuroblasts from the SVZ.

As introduced above, the efficiency of neurogenesis is very low, and protecting the migration and survival of neuroblasts may provide a novel way to improve stroke recovery. The newly generated neuroblasts must migrate along with the newly formed microvessels (Ruan et al., 2015) toward the infarction under the direction of chemo-attractants such as SDF-1 and MCP-1 (Schonemeier et al., 2008). However, oxidative stress, inflammatory factors/reactions, and ischemic conditions can cause neuroblast death, leading to low efficiency of SVZ neurogenesis after stroke (Arvidsson et al., 2002). PDGFRs are expressed mainly on astrocytes, pericytes and neural stem cells, which are the major components of the neurovascular unit. The PDGFR signaling pathway is pivotal to the regulation of several neurovascular functions that are necessary for central nervous system homeostasis under normal or pathologic conditions (Gautam et al., 2016). For example, PDGFs have a significant role in blood vessel formation in ischemic diseases (Cao et al., 2003); the PDGF-CC-PDGFR α signaling pathway, which is regulated by small brain arterioles and astrocytes, can promote BBB permeability after stroke; and PDGFR β is the key signaling pathway that regulates the recruitment, migration, functioning, and survival of pericytes during physiologic and pathologic conditions (Sweeney et al., 2016). In ischemic stroke, both astrocytes and pericytes participate in angiogenesis and local immunoregulation and are important sources of neuroprotectants. But how the PDGFR signaling pathway affects migration and survival of neuroblasts from the SVZ after ischemic stroke and the underlying mechanisms are still unknown.

Neurogenesis within the SVZ is immediately activated by acute ischemic stroke in rodents, reaches a peak on days 7–10, and lasts for over 4 weeks (Parent et al., 2002). To study the role of the PDGFR signaling pathway in the migration of SVZ neuroblasts after stroke, we treated MCAO mice with pan-PDGFR inhibitor crenolanib at the acute stage of stroke (days 1–3), when neurogenesis has just been activated and neuroblast migration has not yet begun. We found that crenolanib significantly delayed recovery of neurologic function and decreased the number of DCX-positive cells that had migrated from the SVZ on day 7 after MCAO. Previous studies have indicated that a majority of DCX-positive cells do not express PDGFR α or PDGFR β , but knocking out PDGFR β gene can increase cell death of neurospheres in vitro (Ishii et al., 2008, Chapman et al., 2015). We also proved that majority of neuroblasts do not express PDGFR β , indicating that crenolanib may interfere with neuroblast function in an indirect way. To investigate potential mechanisms, we explored the angiogenesis and level of chemo-attractants in the ischemic hemisphere. We found that downregulation of the PDGFR signaling pathway significantly decreased the number of proliferative vessels and levels of VEGF, SDF-1, and MCP-1 in the peri-infarct area. These results indicate that PDGFR has a positive role in regulating local angiogenesis and lesion-derived chemo-attractants, which are important for SVZ neuroblast migration after stroke.

To study the role of the PDGFR signaling pathway in survival of SVZ neuroblasts, we treated MCAO mice with crenolanib at the sub-acute stage of stroke (days 7 to 9), when some neuroblasts have migrated to the peri-infarct area. We found that apoptosis of neuroblasts in the ischemic border was significantly increased in crenolanib-treated mice on

day 14 after stroke. The crenolanib-treated MCAO mice also had more activated microglia and lower levels of IL-10, BDNF, and NT-3 in the peri-infarct region, indicating that PDGFR signaling has a positive role in regulating local immunoreactions and neuroprotective chemokines, which are important for the survival of SVZ neuroblasts after stroke.

Our study further showed that crenolanib treatment on days 1–3 or 7–9 after MCAO significantly increased the apoptosis of pericytes (PDGFR β /cCasp3-positive cells) and decreased pericyte/vascular coverage in the peri-infarct area, but had no effect on apoptosis of astrocytes, indicating that the PDGFR signaling pathway may be more important for pericyte function after acute ischemic stroke. Pericytes are a major component of the neurovascular unit and play essential roles in the maintenance of BBB function and regulation of angiogenesis. They are also a major source of neuroprotectants (Oztop-Cakmak et al., 2016, Sweeney et al., 2016), such as VEGF, SDF-1, BDNF, and NT-3, which are all vital for the migration and survival of neuroblasts. Upregulation of pericyte function may be a promising therapeutic method for treating ischemic diseases. Studies on myocardial infarction in mice showed that transplantation of pericytes or pericyte progenitor cells can promote local angiogenesis, reduce cell apoptosis, increase blood flow, and promote functional and structural recovery (Katare et al., 2011, Chen et al., 2013). Together with the results of our study, these findings indicate that the PDGFR β signaling pathway and pericytes may be therapeutic targets for stroke.

We found that crenolanib treatment of mice in the sham groups had no effect on neurogenesis in the SVZ, angiogenesis, or secretion of chemo-attractants or neuroprotectants in the cortex. These results indicate that the PDGFR signaling pathway may play a more positive role in the migration and survival of SVZ neuroblasts after acute ischemic stroke than under normal physiologic conditions.

The two subtypes of PDGFR have opposite roles after acute stroke: PDGFR α signaling contributes to tPA-induced BBB impairment after ischemic stroke and to BBB leakage after intracerebral hemorrhage (Su et al., 2009, Ma et al., 2011). Blocking the PDGFR α signaling pathway with pan-PDGFR inhibitor imatinib in the acute phase of stroke reduced BBB permeability and improved neurologic performance in rodents with stroke (Rieckmann, 2008, Su et al., 2008, Su et al., 2009, Ma et al., 2011). In contrast to PDGFR α , PDGFR β has positive effects after stroke. It is expressed mainly on pericytes and is the key signaling pathway for regulating their function (Sweeney et al., 2016). Mice deficient in PDGFR β completely lack brain pericytes and have increased BBB permeability that results in central nervous system microhemorrhages (Obermeier et al., 2013). Pharmacologic inhibition of PDGFR β caused pericyte depletion and hemorrhage in rodents (Hall et al., 2016). Those findings indicate that blocking PDGFR α alone in the acute phase, without inhibiting the function of PDGFR β , might provide an effective therapy for stroke. However, no specific PDGFR α inhibitor is currently available. Considering the protective roles of PDGFR β signaling pathway after stroke, we conducted this study to examine the possible side-effects of PDGFR inhibition in MCAO mice. We used crenolanib to inhibit PDGFR signaling pathway. According to the drug instruction and previous studies (Buchdunger et al., 1995, Heinrich et al., 2012, Dai et al., 2013), imatinib inhibits pan-PDGFR with an IC₅₀ of 100nM

and crenolanib inhibits PDGFR α/β with IC₅₀ of 2.1nM/3.2nM, crenolanib has a higher potency than imatinib in inhibiting PDGFR α , indicating that crenolanib is more selective for inhibiting PDGFR α/β than imatinib. We found 3 days of treatment with 15 mg/kg crenolanib decreased neurogenesis and angiogenesis, and had negative impact on lesion-derived chemo-attractants, local microenvironment and BBB permeability in MCAO mice. The drug type, dosage and administration time may be the reasons for the discrepancy between this and previous studies, which indicates that the PDGFR inhibition after acute ischemic stroke needs carefully research considering its side-effects on PDGFR β signaling pathway.

The major limitation of this study was the use of a pharmacologic inhibitor in animals. Potential systemic effects or off-target effects might be responsible for some of the reported results. In addition, further exploration is warranted to verify potential causal relationships between the migration and survival of neuroblasts and other results from this study.

In conclusion, we found that the PDGFR signaling pathway can promote the migration and survival of neuroblasts from the SVZ after stroke, PDGFR signaling pathway can also affect angiogenesis, lesion-derived chemo-attractants and local microenvironment, which are of importance in the stroke-induced neurogenesis. However, the multiple mechanisms that contribute to paracrine effects and cellular interactions with the PDGFR signaling pathway need additional research.

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List of abbreviations

BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
cCasp3	cleaved-caspase 3
DCX	doublecortin
IL-10	interleukin-10
MCAO	middle cerebral artery occlusion
MCP-1	monocyte chemotactic protein 1
mNSS	modified neurologic severity score
NT-3	neurotrophin-3

PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
SVZ	subventricular zone
SDF-1	stromal cell-derived factor-1
tPA	tissue plasminogen activator
VEGF	vascular endothelial growth factor

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Research Highlights

- We used crenolanib to study the role of PDGFR pathway in MCAO mice.
- Crenolanib interfered migration and survival of neuroblasts from SVZ to infarction.
- Crenolanib decreased angiogenesis and chemo-attractants secretion in MCAO mice.
- Crenolanib activated microglia and decreased neuroprotectants' level in MCAO mice.
- Crenolanib accelerated the apoptosis of pericytes in peri-infarction of MCAO mice.

Figure 1. PDGFR signaling pathway promotes migration and survival of neuroblasts from the SVZ after stroke

(A) Immunofluorescence staining of neuroblasts (DCX⁺) in the peri-infarct region on day 7 after MCAO. Images are shown at 100× magnification; scale bar=50 μm. (B) Quantification showed that fewer neuroblasts had migrated from the SVZ in the crenolanib-treated MCAO mice than in the vehicle-treated mice on day 7 after MCAO. **p*<0.05 vs. MCAO+vehicle group; *n*=8/group. (C–D) Immunofluorescence staining of PDGFRβ on neuroblasts (DCX⁺) within SVZ (on day 3) or in peri-infarction (on day 14) on after MCAO. Images are shown at 400× magnification; scale bar=50 μm. (E–F) Immunofluorescence staining of apoptotic pericytes (PDGFRβ⁺/cCasp3⁺) in the peri-infarct region on day 14 after MCAO. Images are shown at 200× magnification; scale bar=50 μm. (G) Quantification showed that MCAO mice treated with crenolanib had more apoptotic pericytes than did those treated with vehicle. **p*<0.05 vs. MCAO+vehicle; *n*=8/group. (H) Modified neurologic severity score (mNSS) testing showed that crenolanib treatment of mice on days 1 to 3 after MCAO significantly delayed recovery of neurologic function compared to that of mice treated with vehicle. Significant differences in neurologic function began to be apparent at 2 weeks and continued through at least 5 weeks. **p*<0.05 vs. MCAO+vehicle group; *n*=10/group.

Figure 2. PDGFR signaling pathway promotes angiogenesis and chemo-attractant expression (A–C) Immunofluorescence staining of proliferative vessels (CD31⁺/BrdU⁺) in the peri-infarct region on day 7 after MCAO. CD31: green, BrdU: red. Images are shown at 200× magnification; scale bar=50 μm. (D) Quantification showed that crenolanib treatment on days 1–3 significantly decreased the number of proliferative vessels in the peri-infarct area on day 7 after stroke. * $p < 0.05$ vs. MCAO+vehicle group; $n = 8$ /group. (E) ELISA analysis of ischemic border on day 7 after MCAO showed that crenolanib treatment on days 1–3 decreased the average level of VEGF in MCAO mice, but the change was not statistically significant. $n = 8$ /group. (F–G) Immunofluorescence staining of SDF-1 and MCP-1 in the peri-infarct region on day 7 after MCAO. The white lines show the brain boundaries. Images are shown at 100× magnification; scale bar=25 μm. (H–I) ELISA analysis of SDF-1 and MCP-1 on day 7 after MCAO showed that levels of SDF-1 and MCP-1 in the ischemic border were lower in MCAO mice treated with crenolanib (on days 1–3) than in MCAO mice treated with vehicle. * $p < 0.05$ vs. MCAO+vehicle group; $n = 8$ /group.

Figure 3. PDGFR signaling pathway regulates local microenvironments

(A) Immunofluorescence staining of activated microglia (CD68⁺) in the peri-infarct region on day 14 after MCAO. Images are shown at 200× magnification; scale bar=50 μm. inf, infarct. (B) Quantification showed that crenolanib treatment (on days 7–9) significantly increased the number of activated microglia in the peri-infarct area on day 14 after stroke.

* $p < 0.05$ vs. MCAO+vehicle group; $n = 8$ /group. (C–E) ELISA analysis showed that MCAO mice treated with crenolanib on days 7–9 after MCAO had lower levels of BDNF (C), NT-3 (D), and IL-10 (E) than did vehicle-treated mice on day 10. * $p < 0.05$ vs. vehicle group; $n = 8$ /group.

Figure 4. Blocking PDGFR signaling pathway with crenolanib causes pericyte apoptosis
(A) Immunofluorescence staining of apoptotic pericytes (PDGFR β^+ /cCasp3 $^+$) in the peri-infarct region on day 7 (A1) or day 14 (A2) after MCAO. Images are shown at 200 $\times$ magnification; scale bar = 25 μ m. **(B)** Immunofluorescence staining of pericyte/vessel (PDGFR β^+ /Lectin) coverage in the peri-infarct region on day 7 (B1) or day 14 (B2) after MCAO. Images are shown at 200 $\times$ magnification; scale bar=25 μ m. **(C)** Immunofluorescence staining of apoptotic astrocytes (GFAP $^+$ /cCasp3 $^+$) in the peri-infarct region on day 7 (C1) or day 14 (C2) after MCAO. Images are shown at 200 $\times$ magnification; scale bar = 25 μ m. **(D)** Quantification showed that MCAO mice treated with crenolanib on days 1–3 or 7–9 had more apoptotic pericytes than did the corresponding vehicle-treated mice. * p <0.05 vs. MCAO+vehicle group; n =8/group. **(E)** Quantification showed that MCAO mice treated with crenolanib on days 1–3 or 7–9 had a lower ratio of pericytes to vessels than did the corresponding vehicle-treated mice. * p <0.05 vs. MCAO+vehicle group; n =8/group. **(F)** Quantification showed that MCAO mice treated with crenolanib on days 1–3 or 7–9 had a similar number of apoptotic astrocytes as the corresponding vehicle-treated mice. * p <0.05 vs. MCAO+vehicle group; n =8/group.

Figure 5. Blocking the PDGFR signaling pathway with crenolanib increases blood-brain barrier permeability

(A) Nessler staining of brain sections from MCAO+vehicle (1–3 d) and MCAO+crenolanib (1–3 d) groups on day 3 after MCAO. (B) Quantification showed that crenolanib did not affect the infarct volume. $p > 0.05$, $n = 8$ /group. n.s., not significant. (C and D) Immunofluorescence staining of occludin and albumin (ALB) in the peri-infarct region on day 3 after MCAO. Images are shown at 200 $\times$ magnification; scale bar = 50 μ m. (E) Western blot analysis of occludin and albumin in the peri-infarct region on day 3 after MCAO. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. (F and G) Quantification of band densities showed that treatment of MCAO mice with crenolanib (1–3 d) significantly decreased occludin level and elevated albumin level in the peri-infarct region compared to corresponding levels in the vehicle control mice. $*p < 0.05$ vs. MCAO+vehicle group; $n = 8$ /group.