Hirudin promotes angiogenesis by modulating the cross-talk between p38 MAPK and ERK in rat ischemic skin flap tissue

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ABSTRACT

Hirudin’s ability to increase angiogenesis in ischemic flap tissue and improve the flaps survival has been demonstrated in our previous studies. However, the knowledge about hirudin functional role in angiogenesis is still limited. In the present study, we investigate the effects of locally injected hirudin on the expression of VEGF, endostatin and thrombospondin-1 (TSP-1) using rat model. Caudally based dorsal skin flaps were created and were treated with hirudin or normal saline. Result showed that the flap survival was improved by hirudin treatment relative to the control. Treatment of flaps with hirudin exerted significant angiogenic effect as evidenced by increased VEGF expression and reduced endostatin and TSP-1 production (p < 0.01), and promoted neovascularization (microvascular density, p < 0.01). Moreover, hirudin treatment increased the ERK1/2 phosphorylation, while attenuated the phosphorylation of p38 MAPK, and the addition of thrombin could reverse these effects of hirudin on ERK1/2 and p38 MAPK activity. The MEK inhibitor blocked the hirudin-induced VEGF expression, and the p38 MAPK inhibitor attenuated the thrombin-induced TSP-1 expression. Furthermore, a specific inhibitor of p38 MAPK activates ERK1/2 in ischemic flaps, suggesting that cross-talk between p38 MAPK and ERK might exist in rat ischemic flap tissue. Moreover, either the hirudin or SCH77297 (PAR1 antagonist) could attenuate the p38 MAPK phosphorylation and increases the ERK1/2 phosphorylation, indicating that the cross-talk between p38 MAPK and ERK1/2 modulated by thrombin/PAR1 signaling may participate in the process of hirudin-stimulated ERK1/2 signaling. In conclusion, these observations suggest that hirudin exerts its angiogenesis effect by regulating the expression of angiogenic and antiangiogenic factors via a cross-talk of p38 MAPK-ERK pathway.

1. Introduction

Random skin flap is common for repairing wound, reconstructing the function and improving skin appearance. If a high length to width ratio random skin flap is raised, the distal tissue of the flap inevitably subject to ischemia and subsequent necrosis which often results in partial loss of the flap.

Our previous studies have demonstrated that topical application of hirudin in ischemic flap could stimulate neovascularization and improve flap survival through increase the expression of vascular endothelial growth factor (VEGF) mRNA (Guo et al., 2013). However, the molecular pathways that regulate the process of hirudin-induced VEGF expression in ischemic flap tissue remain unclear. Mitogen-activated protein kinases (MAPKs) play a critical role in cellular proliferation and angiogenesis (Zhan et al., 2003; Zhang and Liu, 2002). The extracellular signal-regulated kinases (ERK) and p38 MAPK are the best known members of MAPKs. The best-characterized function of ERK and p38 MAPK is their regulation of transcription factors such as c-Jun and AP-1 (Cowley et al., 1994). The AP-1 or c-Jun/AP-1 dimer was described as a DNA-binding activity that recognized the VEGF promoter which contains the AP-1 binding site (Xu et al., 2002; Lee et al., 2006). Increase the AP-1 binding activity by MAPKs activation might lead to the activity of VEGF gene transcription. These studies provide a possibility that hirudin might regulate VEGF expression through its modulation of ERK and p38 MAPK activity.

Angiogenesis is a complex process and regulated by a balance of angiogenic and antiangiogenic factors. Endostatin, a 20 kDa
protein, is a specific inhibitor of endothelial cell proliferation, migration and tube formation. The antiangiogenic effect of endostatin is associated with blockade of VEGF signaling by binding to the receptors α3β1 or glycoparian 1/4 on endothelial cells (Abdollahi et al., 2004). Thrombospondin-1 (TSP-1) is the first identified endogenous angiogenesis inhibitor (Bagavandoss and Wilks, 1990). It inhibits angiogenesis by directly interacting with VEGF (Kaur et al., 2010) or by activating CD36 and inducing apoptosis in endothelial cells (Jimenez et al., 2000; Isenberg et al., 2009). The synthesis of both endostatin and TSP-1 are stimulated by thrombin (Ma et al., 2001: Ma et al., 2005; Baenziger et al., 1971). Therefore, we hypothesize that hirudin, a specific thrombin inhibitor, might attenuate the inducing effect of thrombin on endostatin and TSP-1 expression.

In the present study we tested the hypothesis that hirudin induces angiogenesis in ischemic flap tissue by regulate the balance of angiogenic and antiangiogenic factors using a vivo model. We also determined how the ERK and p38 MAPK signaling play a role in the hirudin associated angiogenesis.

2. Materials and methods

2.1. Animals and reagents

Sprague-Dawley rats (250–300 g body wt) were purchased from Guangxi Medical University Laboratory Animal Center (Guangxi, China). The managements of experimental animals in this research were according to Regulations for the Administration of Affairs Concerning Experimental Animals (Approved by the State Council of China and promulgated by Decree No. 2 of the State Science and Technology Commission on November 14, 1988).

Lyophilized hirudin powder (Patent No. ZL03113566.8, Lot No. KK-001) was provided by Nanning JinXueHuang Bioengineering Co., Ltd (Guangxi, China). The VEGF (ab46154), the CD34 (ab81289), the thrombin (ab92621) and the TSP-1 (ab85762) antibody were purchased from Abcam. The immunohistochemical staining kit was purchased from Beijing Golden Bridge Biotechnology Co., Ltd (Beijing, China). The phosho-p44/42 MAPK (Thr202/Tyr204) Rabbit mAb (cat #4370), the p44/42 MAPK Rabbit mAb (cat #4695), the phosho-p38 MAPK (Thr180/Tyr182) Rabbit mAb (cat #4511), the p38 MAPK Rabbit mAb (cat #8690) and the GAPDH Rabbit mAb (cat #5174) were from Cell Signaling Technology. The secondary antibody anti-rabbit IRDye-800CW were purchase from LI-COR Biosciences. The MEK inhibitor (PD98059), the p38 MAPK inhibitor (SB203580) and the pazopanib were purchased from Selleckchem. The SCH779797 was purchased from Santa Cruz Biotechnology.

2.2. Flap creation and treatment

Two caudally based dorsal skin flaps were created in each rat. Briefly, rats were anesthetized by intraperitoneal injections of xylazine (10 mg/kg) and ketamine (50 mg/kg). The dorsal hair was removed, and the skin was sterilized with betadine. Two caudally based dorsal skin flaps (7.5 cm long and 1.5 cm wide) were created on the either sides of dorsal midline respectively. The entire flap was undermined below the level of the panniculus carnosus and then sutured in situ.

The flaps were injected with hirudin (hirudin group) or normal saline (control group) immediately after surgery and again on postoperative day 1, 2, 3 and 4. In hirudin group, 2 antithrombin units (ATU) hirudin (0.4 mL) were injected into each flap. In the control group, isotonic normal saline (0.4 mL) was injected into each flap. The injections were performed with a needle at the flap hypodermic level.

2.3. Histopathological assessment and Immunohistochemistry

The samples were preserved in 10% formalin solution. Transverse sections were taken at the distal part of the flaps. Paraffin-embedded and 5 μm sectioned tissue samples were stained with hematoxylin and eosin and assessed under a light microscope.

For immunohistochemistry staining, tissue sections were pretreated with citrate buffer and then incubated with CD34 monoclonal antibody for 2 h at room temperature. A biotin-conjugated secondary antibody was used at an appropriate dilution and applied at 20–37°C for 20 min incubation. Sections were then incubated in DAB-brown for 3 min to produce brown reaction product. Digital images of tissue sections were captured using an Olympus BX53 light microscope. With regard to vascularization, 3 most vascularized areas were chosen for microvessel counting after CD34 staining. Microvessel counts were performed at 200×, and microvessel density (MVD) was defined as the mean count of microvessels per 0.74 mm² field area.

2.4. Western blot

Tissues were grinded and lysed in a buffer (0.5% Nonidet P-40, 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1 mM Na3VO4) containing protease (1 mM PMSF) and phosphatase inhibitors (PhosSTOP, Roche). The total protein concentration was measured by BCA assay (BOSTER, China) according to the protocols. Proteins were separated on a 10% SDS-PAGE and followed by transfer onto a polyvinylidene difluoride membrane (Bedford, USA) for 1.5 h at 100 mA. Membranes were incubated in 3% BSA/TBS-T for 1 h at room temperature. Primary antibodies were used at an appropriate dilution and applied overnight incubation at 4°C. Subsequently, the membrane was incubated with fluorescent-conjugated secondary antibody for 1 h. Blots were analyzed using the Odyssey imaging system (LI-COR Biosciences).

2.5. Flap survival measurement

Photographs of the flaps were taken postoperatively at day 6 for evaluate the viable flap area and the necrotic flap area. The flap survival rate was calculated with the formula: Flap survival rate = (Viable flap area/total flap area) × 100%.

2.6. Statistical analysis

All data were expressed as mean ± SD. The data between groups, statistical significance was determined by Compare Student’s t-test or One-Way ANOVA. Statistical analyses were performed using SPSS version 13.0, and a value of p < 0.05 was considered statistically significant.

3. Results

3.1. Flap observation and determination of flap survival rate

At the time of operation, the appearance of the flaps was not different between the two groups. On the 4th day after operation, the distal parts of the flaps in control group were turning dark purple or cyanotic, whereas the hirudin group show minimal changes. By postoperative day 6, the necrotic area had stabilized with clear boundaries between the viable and necrotic areas (Fig. 1A). The appearance of necrotic area was black color and hard. After calculation, the flap survival rate in hirudin group (90 ± 5.8%) was significantly larger than that in the control (62 ± 7.1%) group.
thickened and with a normal structure among the hirudin-treated flaps (Fig. 1B).

In sections from each group, maximal microvessel density was observed in the dermal and subdermal layers. At postoperative day 4, MVD in sections from hirudin group was significantly higher than that in the control (mean ± SD: 31.50 ± 5.68 vs. 15.90 ± 5.64 per field). At postoperative day 6, MVD in sections from hirudin group was 35.90 ± 6.76 per field, whereas massive necrosis and no microvessel were found in sections from control group (Fig. 1C).

3.3. Effect of hirudin on VEGF, TSP-1 and endostatin expression

We first test whether thrombin was upregulated in ischemic flap tissue. The expression of thrombin in the distal part of the flaps from postoperative day 0–6 was detected by using Western blot. We observed that the production of thrombin was upregulated in ischemic flap tissue, and hirudin treatment could limit the sudden increase of thrombin at day 1 and day 2 (Fig. 2B).

To investigate the effect of hirudin on angiogenesis, we next detected the expression of VEGF, endostatin and TSP-1 in the distal part of the flaps. We observed that the expression of VEGF in both two groups was elevated at postoperative day 1, but the level of VEGF in hirudin group was 4-fold higher than that in the control group (p < 0.01). From postoperative day 2–6, the expression of VEGF maintain in high lever in hirudin-treated flaps (p < 0.01, Fig. 2C). This result is consistent with the findings of our previous study (Guo et al., 2013).

In hirudin group, the expression of endostatin was decrease on postoperative day 1, 2 and 4 compared with control group (p < 0.05), but it showed a rising trend and even reaching the same level as the control group at day 6 (p > 0.05, Fig. 2D). Moreover, in flaps treated with hirudin there was a dramatic decrease (p < 0.01) in TSP-1 production from postoperative day 1–6 (Fig. 2E). These data indicate that the hirudin-induced angiogenesis in ischemic flaps are also associated with decrease of TSP-1 and endostatin expression.

3.4. Effect of hirudin on the activation of ERK and p38 MAPK

To determine whether ERK1/2 and p38 MAPK activity could be modulated by hirudin treatment, we examined the status of ERK1/2 and p38 MAPK phosphorylation in ischemic flap tissue. The data in Fig. 3A demonstrate that phosphorylation of ERK1/2 was first elevated in both two groups at day 1 after operation. The phosphorylation level of ERK1/2 in hirudin-treated flaps at each time point was significantly higher then that in the control (p < 0.01). The phosphorylation of p38 MAPK in flaps from each group was significant increased after operation, but the activation of p38 MAPK in hirudin-treated flaps was lower than that in the control (p < 0.01, Fig. 3B). Taken together, these results demonstrate that topical application of hirudin increased ERK1/2 phosphorylation, while attenuated the phosphorylation of p38 MAPK.

Hirudin regulates cellular function through inhibition of thrombin signaling (Karabiyikoglu et al., 2004; Feutz et al., 2008). Therefore, we further investigated whether thrombin treatment could reverse the effects of hirudin on ERK1/2 and p38 MAPK activity. After 2 days of treatment with 2 ATU of hirudin, flaps were injected subcutaneously with thrombin (0.5–2.0 Units). Samples were harvested 3 h after thrombin treatment. The results showed that thrombin induced a dose-dependent activation of p38 MAPK but simultaneously reduced ERK1/2 activity in hirudin-treated flap tissue (p < 0.01, Fig. 3C). The results suggest the possibility that hirudin regulates the phosphorylation status of ERK1/2 and p38 MAPK through mediates the thrombin signaling.
Fig. 2. (A) Detection of thrombin, VEGF, endostatin and TSP-1 in ischemic flap tissue by western blot analysis. GAPDH was used as an internal control and the expression of thrombin, VEGF, endostatin and TSP-1 protein was quantified as densitometry value analyzed. The protein density of the thrombin (B), VEGF (C), endostatin (D) and TSP-1 (E) for each group is displayed. Each data point represents the mean ± SD. *p < 0.05 and **p < 0.01 vs. the control. n = 5 for each group.

Fig. 3. Effect of hirudin on the p38 MAPK and the ERK1/2 activity. The phosphorylation of ERK1/2 (A) and p38 MAPK (B) were evaluated by western blot. Hirudin treatment leads to an increase in ERK1/2 phosphorylation, while decreased the phosphorylation of p38 MAPK. **p < 0.01 vs. the control, n = 5 for each group. (C) Thrombin treatment reversed the opposing effects of hirudin on ERK1/2 and p38 MAPK activity. *p = 0.01 and ##p < 0.01 vs. the activation of ERK1/2 and p38 MAPK of 0 unit thrombin group, respectively. Statistical significance was determined using One-Way ANOVA. n = 5 for each group.
3.5. Hirudin inhibit TSP-1 and endostatin production via thrombin signaling

To examine the possibility that thrombin treatment could reverse the attenuating effect of hirudin on the TSP-1 and endostatin expression, flaps were injected subcutaneously with thrombin (0.5–2.0 Units) after 3 h of hirudin treatment. Samples were harvested 3 h after thrombin treatment. The results showed that TSP-1 and endostatin exhibited dose-dependent thrombin-induced expression (Fig. 4A and B). To understand whether p38 MAPK function upstream of TSP-1 and endostatin synthesis, 100 μM of SB203580 was injected subcutaneously 30 min before thrombin (2.0 Units) treatment. The results showed that treatment of flaps with SB203580 produced a significant inhibition of p38 MAPK activity (p < 0.01, Fig. 4C). Inhibition of p38 MAPK activation resulted in significant decrease of TSP-1 expression, but not endostatin (Fig. 4A and B), suggesting that p38 MAPK function upstream of thrombin-induced TSP-1 synthesis.

We next test whether the stimulating effect of thrombin on TSP-1 and endostatin expression could be blocked by hirudin. Flaps were injected subcutaneously with 2 units of thrombin for 3 h and followed by the injection of hirudin (0.5–2.0 ATU) to neutralize thrombin. As demonstrated in Fig. 4 D–F, hirudin abolished the stimulating effect of thrombin on the TSP-1 and the endostatin expression and the p38 MAPK activity (p < 0.01). Taken together, these data suggest that hirudin suppresses the expression of TSP-1 and endostatin via abolish the thrombin signaling, and the effects of thrombin-induced TSP-1 expression involved an activation of p38 MAPK.

3.6. Inhibition of p38 MAPK stimulates ERK1/2 activity

Previous studies have confirmed that p38 MAPK-ERK cross-talk modulates ERK activation status (Westermark et al., 2001; Liu and Hofmann, 2004). We, therefore, test whether hirudin modulates the phosphorylation status of ERK via mediating the cross-talk between p38 MAPK and ERK. First, we examined whether the cross-talk of p38 MAPK-ERK exists in rat ischemic flap tissue. After 3 h of SB203580 treatment, the activation of ERK1/2 was increased significantly (p < 0.01, Fig. 5A and B), indicating that ERK1/2 activation is associated with p38 MAPK in ischemic flap tissue. Because hirudin treatment caused an increase in ERK1/2 phosphorylation, we used the hirudin-treated flap model to test whether the cross-talk between p38 MAPK and ERK1/2 is bidirectional. The p38 MAPK phosphorylation was examined after 3 h of treatment with MEK inhibitor (PD98059). However, inhibition of the MEK/ERK pathway by PD98059 had no significant effect on p38 MAPK phosphorylation (p > 0.05, Fig. 5A and B). These data suggests that a unidirectional cross-talk between p38 MAPK and ERK might exist in rat ischemic flap tissue.

Protease-activated receptor 1 (PAR1) has been considered to be the most important thrombin receptor and it plays a major role in thrombin signal transduction (Coughlin, 2000). To understand whether the status of p38 MAPK phosphorylation was modulated by thrombin-induced PAR1 stimulation, 50 μM of SCH779797 (a selective PAR1 antagonist) were injected into the flaps subcutaneously. The western blot assay suggested that inhibition of PAR1 caused significant decreased phosphorylation of p38 MAPK (p < 0.01, Fig. 5C). In contrast, inhibition of PAR1 increased the phosphorylation of ERK1/2. These results were similar to the observations in hirudin-treated flaps. In addition, treatment of 50 μM SCH779797 was more effective than 2.0 ATU hirudin to decrease the p38 MAPK activity (p < 0.01), but the level of ERK1/2 activity in 50 μM SCH779797-treated group was still lower than that in the hirudin-treated group (p < 0.01, Fig. 5C–E), indicating that hirudin increase ERK1/2 phosphorylation is not totally through the inhibition of thrombin/PAR1-induced p38 MAPK activation and there may be has another pathway participated in this process. To confirm this hypothesis, PAR1 signaling was blocked by 50 μM of SCH779797, and then the flaps were treated with 2.0 ATU of hirudin. Interestingly, although addition of hirudin in SCH779797-treated flaps did not provide a further decrease of p38 MAPK activity (compare to the 50 μM SCH779797 treatment alone), the level of ERK1/2 activity in flap treated with SCH779797 together with hirudin increased to the same level as that of flap treated with 2.0 ATU of hirudin alone (Fig. 5D and E), suggesting that there are another pathways that involved in the hirudin-mediated ERK1/2 activity. In addition, treatment of flaps with selective PAR4 antagonist did not affect the status of ERK1/2 and p38 MAPK phosphorylation in flap tissue under ischemia conditions (data not show). Taken together, these data suggest that thrombin/PAR1 signaling may participate in the regulation of ERK1/2 phosphorylation in ischemic flap tissue and the thrombin inhibitor hirudin may attenuate the thrombin-induced p38 MAPK activation to allow a further phosphorylation of ERK1/2 in ischemic flaps.

3.7. Hirudin-induced VEGF expression is correlated with ERK activity

To understand the dose required to improve VEGF expression, flaps from each group were injected with increasing concentrations of hirudin. The ultimately concentrations of hirudin were 0.05, 0.1 and 2.0 ATU/day in each flaps respectively. On postoperative day 2, total proteins were harvested 180 min following treatment of flaps with the hirudin and were subjected to Western blot analysis. The data showed that hirudin administration induced dose-dependent increases in ERK1/2 activation and VEGF expression (p < 0.01, Fig. 6A and B). We next tested whether treatment of flaps with MEK inhibitor (PD98059) could block the hirudin-induced VEGF expression. After 2 days of treatment with 2.0 ATU/day hirudin, flaps were injected with 100 μM PD98059. We found that the ERK1/2 activation was significant attenuated and the expression of VEGF was significant decreased after 3 h treatment of PD98059 (p < 0.01, Fig. 6B). This result indicates that the activation of ERK1/2 is associated with hirudin-induced expression of VEGF.

VEGF-stimulated activation of the ERK1/2 signaling is required for cell proliferation, whether the increase of ERK1/2 activity was correlated with the upregulation of VEGF needed further investigation. In this experiment, pazopanib (a drug that targets multiple VEGF receptors) was used to inhibit VEGF receptors activity. An approximate 27% decrease of ERK1/2 phosphorylation (p < 0.01) was observed in ischemic flap tissue when 50 mg/kg pazopanib was given (intragastric administration) 3 h following 2 days of hirudin administration (Fig. 6C). However, a higher dose of pazopanib (100 mg/kg) administration did not further decrease the phosphorylation of ERK1/2 as compare to the 50 mg/kg pazopanib group (p > 0.05). The results indicate that the ERK1/2 activity also partially stimulated by the VEGF.

4. Discussion

Our previous studies have found that topical application of hirudin could reduce the local inflammatory response, alleviated capillary permeability and improving blood circulation and angiogenesis, so that it could improve the survival rate of ischemic flaps (Guo et al., 2013; Yin et al., 2012). However, the molecular mechanism that mediates the angiogenic effect of hirudin in ischemic flaps needs further investigations. These experiments show that topical application of hirudin could up-regulate the expression of VEGF and suppress the TSP-1 and endostatin production, and the angiogenic effect of hirudin in ischemic flap
might be mediated by the cross-talk between p38 MAPK and ERK pathways.

Thrombin is the key product of the coagulation system and is produced following injury. The thrombin generation level is associated with ischemia. Evidence indicates that a large amount of thrombin is produced immediately after brain ischemia, and high concentrations of thrombin within ischemic brain tissue is likely to cause more brain injury (Erlich et al., 2000). Moreover, high
A significant increase of ERK1/2 phosphorylation was observed in ischemic flaps treated with SB203580. However, the presence of PD98059 did not alter background p38 MAPK phosphorylation in hirudin-treated flaps. Statistical significance was determined using One-Way ANOVA, and n = 5 for each group. *p < 0.01; **p < 0.01 and ##p < 0.01 vs. the activation of ERK1/2 and p38 MAPK of 2 unit thrombin without SB203580 group, respectively. (C–E) Treatment of flaps with PAR1 antagonist SCH79797 caused decreased phosphorylation of p38 MAPK and increased phosphorylation of ERK1/2. **p < 0.01, n = 5 for each group.

Concentrations of thrombin could result in apoptosis of endothelial cells (Thippegowda et al., 2010). Although production of the thrombin has been associated with cerebral ischemic events, there is little previous evidence implicating this coagulation protein in flap ischemic tissue. In our study, a sustained expression of thrombin was observed in ischemic flap tissue over the entire period of 6 days. This result suggests the possibility that thrombin signaling pathway may be involved in the angiogenic process within ischemic flap tissue.

The endostatin and the TSP-1 are extracellular matrix-associated proteins that inhibit angiogenesis. Bloch has found that endostatin treatment does not affect wound closure, but causes hemorrhage and severe narrowing of vessels in the wound tissue (Nissen et al., 1998). Angiogenesis in some tumors occurs as a result of an imbalance in production between VEGF and endostatin (Hu et al., 2014; Poon et al., 2004). Recent studies have proved that thrombin stimulated endostatin release via activation of PAR4 (Ma et al., 2001; Ma et al., 2005). Our current findings show that the production of endostatin was decreased in hirudin-treated flap tissue and thrombin treatment stimulated endostatin release. However, p38 MAPK inhibitor does not affect thrombin-induced endostatin expression, suggesting that the decrease of the endostatin expression caused by hirudin dose not associated with the p38 MAPK signaling.

TSP-1 was first isolated from thrombin-stimulated human platelets (Baenziger et al., 1971). It is potent endogenous inhibitors
of angiogenesis and inhibits angiogenesis directly by interacting with VEGF in endothelial cell (Lawler and Lawler, 2012). Blockade of the TSP–1 has been shown to promote flap perfusion, survival and angiogenesis in mice (Isenberg et al., 2009). We found that topical application of hirudin led to a significant decrease in TSP–1 expression. It has been demonstrated that the activation of Rho and ERK in endothelial cells are necessary for up-regulation of TSP–1 synthesis induced by thrombin in vitro (Martinez-Sales et al., 2007). However, a wealth of reports has suggested that p38 MAPK function upstream of TSP–1 expression induced by TGF–β (Takekawa et al., 2002). Angiotensin II (Naito et al., 2004) and hypoxia (Ortiz-Masià et al., 2012). In this study, we demonstrated that the p38 MAPK activation and the TSP–1 synthesis were significantly upregulated by thrombin treatment and that these upregulation were inhibited by treatment with the hirudin. Furthermore, the expression of TSP–1 was also clearly decreased in response to p38 MAPK inhibition, in line with the observation that TSP–1 expression is also influenced by p38 MAPK activation status. These results indicate that hirudin suppress TSP–1 expression via inhibiting thrombin/p38 MAPK pathway in ischemic flap tissue.

Experimental studies have indicated that up-regulation of VEGF can induce regional angiogenesis and improve the survival rate of overdimensioned flaps (Machens et al., 2003; Zhang et al., 2000). Administration of VEGF at the distal part of a long random skin flap could stimulate neovascularization (Vourtsis et al., 2012). Our previous work has demonstrated that the expression of VEGF mRNA was promoted by application of hirudin (Guo et al., 2013). Here, as extension, the present study further investigated the signal pathway that might be involved in the induction of VEGF by hirudin. We observed that the phosphorylation of ERK1/2 was remarkable higher in the hirudin-treated flaps, and the addition of hirudin caused a potent dose-dependent increase in ERK1/2 phosphorylation and VEGF production. More importantly, the up-regulation of VEGF by hirudin was significant attenuated by treatment with the MEK specific inhibitor. Taken together, we speculate that the effects of hirudin-induced VEGF expression involved a continuous activation of MEK/ERK pathways. However, ERK1/2 may function either upstream or downstream of VEGF. In our study, inhibition of VEGF receptors caused an approximate 27% decrease of ERK1/2 phosphorylation, suggesting that the secretion of VEGF also contribute to stimulation of ERK1/2 signaling pathways in flap tissue.

Hirudin is a highly specific and potent inhibitor of thrombin, it can binding to thrombin and thereby inhibits thrombin signaling. It has been reported that thrombin activation of PAR1 stimulates p38 MAPK phosphorylation (Grimsey et al., 2014) and inhibition of thrombin by hirudin resulted in decrease of p38 MAPK phosphorylation (Dickfeld et al., 2001). In our study, hirudin treatment stimulates ERK1/2 phosphorylation, whereas inhibits the
activation of p38 MAPK. Furthermore, thrombin treatment could reverse these opposing effects of hirudin on ERK1/2 and p38 MAPK activity. More importantly, it is well described that hirudin can specifically binds to and inhibits the activity of thrombin both in solution and on cell surfaces but not to other plasma proteins (Zoldhyesi et al., 1993). Thus, we speculate that hirudin effects on ERK1/2 and p38 MAPK activity may be mediated through thrombin signaling.

We next investigated whether the ERK1/2 activation induced by hirudin was mediated through a cross-talk of p38 MAPK-ERK in ischemic flap tissue. Our immunoblot analysis revealed that the phosphorylation of ERK1/2 was significantly increased by SB203580 treatment in ischemic flap tissue. Evidences suggest that specific activation of p38 MAPK does exert inhibitory effects on the MEK/ERK signaling in myocytes and NIH 3T3 fibroblasts. Further, SB203580, a specific inhibitor of p38 MAPK, activates ERK (Westermark et al., 2001; Liu and Hofmann, 2004). It was recently shown that p38 MAPK inhibits ERK activity by blocking MEK phosphorylation via activation of protein phosphatase 2 (PP2A) (Junttila et al., 2008). PP2A is an important negative regulator of the ERK signaling, and inhibition of PP2A by OA or Fors enhances H2O2-stimulated ERK activation in ventricular myocytes (Liu and Hofmann, 2004; Yu et al., 2004). However, studies from Shimo demonstrate that MEK inhibitors treatment increased phosphorylation of p38 MAPK, suggesting that the cross-talk between p38 MAPK and ERK can be bidirectional (Shimo et al., 2007). Unfortunately, our result showed that PD98059 (MEK1 inhibitor) treatment failed to reverse the dephosphorylation of p38 MAPK caused by hirudin, demonstrating that the cross-talk between p38 MAPK and ERK1/2 is unidirectional in flap tissue under ischemia conditions.

Furthermore, we found that treatment of flaps with either the thrombin inhibitor hirudin or a PAR1-selective antagonist could attenuate the p38 MAPK phosphorylation and increases the ERK1/2 phosphorylation, indicating that the cross-talk between p38 MAPK and ERK1/2 modulated by thrombin/PAR1 signaling may participate in the process of hirudin-stimulated ERK1/2 signaling. We also found that even when the PAR1 was blocked by PAR1 antagonist hirudin also can cause a slight increase in ERK1/2 phosphorylation. These observations suggest that PAR1 is not the only pathway that plays a critical role in upregulating the phosphorylation of ERK1/2 after hirudin treatment. There are likely other factors that play a role in this program. Our previous study showed that the circulation of over dimensioned random pattern skin flaps could be significantly improved by hirudin (Yin et al., 2014), so we speculate that the secretion of VEGF and the activation of ERK1/2 may also be associated with the increase of blood supply caused by hirudin treatment. Thus, the mechanism of hirudin-stimulated ERK1/2 phosphorylation in hirudin-treated flaps is more complicated than we have expected. To fully understanding the molecular mechanism will require further work, preferably in the in vitro studies.

5. Conclusion

In summary, our study demonstrated that hirudin treatment not only increase VEGF expression but also decrease endostatin and TSP-1 production, which may potentially contribute to the hirudin mediated effects of improving angiogenesis in ischemic flap tissue. Our data also suggest that a cross-talk from p38 MAPK to ERK pathway appears to exist in ischemic flap tissue, and hirudin may exerts its angiogenesis effect via inhibits the thrombin-induced negative cross-talk of p38 MAPK-ERK. This novel pathway may play a crucial role in the improvement of skin flap survival. This study provides the experimental basis for the clinical application of hirudin to prevent the flap ischemia. However, the clinical applications of hirudin still need confirming and supporting by more basic research.

Conflicts of interest

The authors confirm that there are no conflicts of interest.

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