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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 SCH79797 (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 beast 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

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ptotein, 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 receptor$\alpha_5\beta_1$ or glycopican-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 phospho-p44/42 MAPK (Thr202/Tyr204) Rabbit mAb (cat #4370), the p44/42 MAPK Rabbit mAb (cat #4695), the phospho-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 SCH79797 was purchased from Santa Cruz Biotechnology.

2.2. Flap creation and treatment

Two caudally based dorsal skin flaps were created in each rats. 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 within each flap at the 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 were 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 mm^2 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 Na_3VO_4) 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 shown 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.