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Research Article

Vascular endothelial growth factor promotes cardiac stem cell migration via the PI3K/Akt pathway

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ABSTRACT

VEGF is a major inducer of angiogenesis. However, the homing role of VEGF for cardiac stem cells (CSCs) is unclear. In *in vitro* experiments, CSCs were isolated from the rat hearts, and a cellular migration assay was performed using a 24-well transwell system. VEGF induced CSC migration in a concentration-dependent manner, and SU5416 blocked this. Western blot analysis showed that the phosphorylated Akt was markedly increased in the VEGF-treated CSCs and that inhibition of pAkt activity significantly attenuated the VEGF-induced the migration of CSCs. In *in vivo* experiments, rat heart myocardial infarction (MI) was induced by left coronary artery ligation. One week after MI, the adenoviral vector expressing hVEGF165 and LacZ genes were injected separately into the infarcted myocardium at four sites before endomyocardial transplantation of 2×10^5 PKH26 labeled CSCs (50 μL) at atrioventricular groove. One week after CSC transplantation, RT–PCR, immunohistochemical staining, Western blot, and ELISA analysis were performed to detect the hVEGF mRNA and protein. The expression of hVEGF mRNA and protein was significantly increased in the infarcted and hVEGF165 transfected rat hearts, accompanied by an enhanced PI3K/Ak activity, a greater accumulation of CSCs in the infarcted region, and an improvement in cardiac function. The CSC accumulation was inhibited by either the VEGF receptor blocker SU5416 or the PI3K/Ak inhibitor wortmannin. VEGF signaling may mediate the migration of CSCs via activation of PI3K/Akt.

Introduction

An important cause of heart failure is ischemic heart disease, particularly myocardial infarction (MI), which is a primary myocardial disease characterized by loss of cardiomyocytes and an increase in fibroblasts. It has been assumed that any attempt to replace the lost myocytes using cellular therapy would require the introduction of exogenous cells into the myocardium. However, the presence of cardiac stem or precursor cells (CSCs) in adult hearts has recently been reported, and CSCs can differentiate into cardiomyocytes and vascular endothelial cells [1–3]. Furthermore, CSCs directly injected into an infarcted heart *in vivo* have been shown to induce myocardial regeneration. Dawn et al. [3] also reported that, after intracoronary administration, CSCs could target injured myocardium, differentiate into cardiomyocytes and vascular cells, and improve cardiac function. Despite these

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encouraging observations, the mechanism by which CSCs repair the heart remains unclear. In particular, it is not know how CSCs migrate into the infarcted region following MI.

The expression of vascular endothelial growth factor (VEGF) increases in response to myocardial ischemia and promotes vascular repair. VEGF is widely recognized to be a potential therapeutic target for regulating angiogenesis [4]. Interestingly, among the known stem cell-active chemokines, the angiogenic factor VEGF promotes mobilization and recruitment of endothelial and hematopoietic stem cells into the neo-angiogenic sites, thereby accelerating the revascularization process. It does this through interaction with its receptors, VEGFR2 (Flk-1) and VEGFR1 (Flt-1), which are expressed on endothelial and hematopoietic stem cells [5,6]. Furthermore, our previous study showed that VEGF can induce mesenchymal stem cell (MSCs) differentiation into vascular endothelial cells [7], and promote the migration of MSCs *in vitro* [8]. However, whether VEGF is involved in CSC migration remains unknown. PI3K/Akt signaling has been implicated in multiple cellular and organ functions, including growth, cell survival, glucose metabolism, and protein synthesis. Furthermore, PI3K/Akt pathways have been reported to be responsible for the cytokine-induced signaling of cell migration [9].

The purpose of this study was to address the following questions: whether overexpression of VEGF165 resulted in homing of CSCs to the injured myocardium, and whether the VEGF-induced migration of CSCs was mediated by PI3K/Akt signaling pathway.

Methods

Isolation and culture of c-kit$^+$ cells from rat hearts

CSCs were isolated from the hearts of 3-day-old Sprague-Dawley rats with a method described by Beltrami et al. [10] with a minor modification. Briefly, the hearts were removed under aseptic conditions from rats that were overdosed with sodium pentobarbital. The myocardial tissue was cut into 1–2 mm^3 pieces, washed with Ca^{2+}–Mg^{2+}–free phosphate-buffered solution (PBS) to remove the blood and then digested three times for 5 min at 37 °C with 0.2% trypsin (Invitrogen) and 0.1% collagenase IV (Sigma, Milan, Italy). After the enzymatic digestion, a cell suspension was collected and filtered with a strainer (Becton Dickson). The cells were incubated with a rabbit anti-c-kit antibody (Santa Cruz) and separated using sheep anti-rabbit immunomagnetic microbeads (Miltenyi Biotec, Auburn, CA). Small round cells, with most positive for c-kit, were collected. Newly isolated cardiac c-kit$^+$ cells were grown in fibronectin-coated 25 cm^2 culture flasks in DMEM containing 15% fetal calf serum (FCS, Hyclone, USA), 10 ng/mL bFGF, and 10 ng/mL LIF for 3 days (5% CO_2, 37 °C). After recovery, the cells were used for the experiments. To induce differentiation, the cells were cultured in medium containing 15% FCS and 50 ng/mL VEGF (Upstate) in a 24-well system for 7 days.

Transwell migration assay

DMEM (600 μL) plus 2% FCS containing 0.1, 1, 10, 20 or 50 ng/mL VEGF was put into the lower chamber of a 24-well transwell (Millipore, USA). A total of 1×10^5 CSCs in 200 μL of medium were seeded into the upper chamber (pore size, 8 μm). For the inhibition experiment, CSCs were preincubated with a VEGF receptor inhibitor SU5416 (5 μM; SUGEN Inc.) or a PI3K/Akt inhibitor wortmannin (100 nM) for 30 min before seeding. The chamber was then incubated for 12 h at 37 °C in a humidified atmosphere with 5% CO_2. The membrane was removed and its upper surface scraped to remove the unmigrated CSCs. After fixation and staining, the number of CSCs that had migrated to the lower surface of the membrane was counted in 10 random and nonrepeated high-power fields (HPFs) under a light microscope. And the migration index (migration index = stimulated migration / random migration) was calculated [11]. Each assay was performed in triplicate wells.

Construction of adenoviral vector expressing VEGF

Human VEGF165 (hVEGF165) cDNA was obtained by PCR amplification with pfu polymerase (Promega Biotech Co, USA) from pORF-hVEGF165 plasmid (InvivoGen). An "A" was added to the 3′ end of the PCR product by Taq DNA polymerase (Promega Biotech Co, USA) and the PCR product added single "A" was then subcloned into pGEM-T easy vector. hVEGF165 cDNA was released from the plasmid pGEM-T-hVEGF165 by digestion of *Eco*RV and *Xho*I, respectively. The recovered hVEGF165 cDNA was then subcloned into the pShuttle-CMV (Stratagene, USA). Accuracy of the resultant pShuttle-hVEGF165 was confirmed by DNA sequencing. The pShuttle-hVEGF165 was linearized by *Pme*I digestion and CIAP dephosphorylation, and transformed into the competent BJ5183 transfected with pAdeasy-1. After identification, the pAd-hVEGF165 was digested with *Pac*I and transfected into AD293 cells (Stratagene, USA) with cationic liposome to package recombinant adenovirus Ad-VEGF. Ad-VEGF was propagated by repeated rounds of infection of AD293 cells with supernatant of the recombinant adenovirus. Ad-VEGF was purified with CsCl density gradient ultracentrifugation. The titer of Ad-VEGF was 2.4×10^{12} pfu/ml. The control adenoviral vector expressing β-galactosidase was kindly provided by Dr. JN Wang (Institute of Clinical Medicine, Yunyang Medical College).

Model of myocardial infarction and Ad-VEGF delivery

All procedures in the present study were performed in accordance with the Guidelines of the Hubei Council of Animal Care and approved by the Animal Use Subcommittee at the Yunyang Medical College, China.

Male Sprague-Dawley rats were anesthetized by ketamine (50 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). MI was achieved by the ligation of the left anterior descending coronary artery. Successful ligation of coronary was verified by color change in the ischemic area and ECG lead I and aVL S–T segment elevations after occlusion. One week after MI, the animals received an injection of 5.0×10^9 pfu Ad-VEGF or Ad-LacZ (0.2 ml) at four sites (0.05 ml per site) in each infarcted heart. Two injection sites were in the peri-ischemic area, and two, in the ischemic area.

Cell labeling and endomyocardial transplantation

For endomyocardial transplantation, the cultured CSCs were labeled with a cell tracker dye PKH26 according to the manufacturer's instructions (PKH26 Red Fluorescent Cell Linker Kit; Sigma, USA). A total of 2×10^5 PKH26-labeled cells (50 μL) were injected