Thrombosis Research 133 (2014) 590–598

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

MiR-150 enhances the motility of EPCs *in vitro* and promotes EPCs homing and thrombus resolving *in vivo*

Wenbin Wang [1], Chenglong Li [1], Wendong Li, Lingshang Kong, Aimin Qian, Nan Hu, Qingyou Meng *, Xiaoqiang Li *

Department of Vascular Surgery, the Second Affiliated Hospital of Soochow University, Suzhou, 215000, China

ARTICLE INFO

Article history:
Received 13 October 2013
Received in revised form 2 December 2013
Accepted 30 December 2013
Available online 5 January 2014

Keywords:
miR-150
endothelial progenitor cells
migration
c-Myb
thrombus resolution

ABSTRACT

Introduction: Deep venous thrombosis (DVT) is one of the common peripheral vascular diseases. The recruitment and migration of bone marrow-derived endothelial progenitor cells (EPCs) to the sites of venous thrombus are necessary in the process of thrombus organization and recanalization. Our objective was to investigate the functional role of miR-150 in rat EPCs and its potential application in deep venous thrombosis.
Materials and Methods: Rat EPCs were cultured and transfected with miR-150 mimics and inhibitor. Wound healing assay, transwell migration assay and matrigel tube formation assay were performed to elucidate the effect of miR-150 of rat EPCs. Lentiviral construct expressing miR-150 was transfected into EPCs and the EPCs were injected to rat models of DVT. The rats were sacrificed on the day of 7 and 14 after the transplantation and the histological study was performed. Luciferase reporter assay and Western blot were performed to evaluate rat miR-150 regulates the expression of c-Myb.
Results: MiR-150 significantly promoted the migration and tube formation ability of EPCs *in vitro* and enhanced EPCs' homing, organization and resolution ability *in vivo*. Overexpression of miR-150 significantly reduced the protein level of c-Myb and repressed the activity of a luciferase reporter containing both of the two predicted miR-150 binding sites in c-Myb 3'-UTR, indicating that c-Myb may be a miR-150 target gene.
Conclusion: MiR-150 enhanced the migration, tube formation, homing, thrombus recanalization and resolution ability of rat EPCs. Restoring miR-150 in EPCs revealed potential application in DVT therapy.

Introduction

Deep venous thrombosis (DVT) is a common peripheral vascular disease. DVT may lead to the abnormal swelling and ulceration of lower limb, post-thrombotic syndrome (PTS) or even pulmonary embolism (PE) which can lead to the sudden death of patients. It was reported that the incidence of venous thromboembolism is one thousand per year [1]. Currently, the clinical treatment for DVT is using of anticoagulants to reduce the incidence of PE, PTS and recurrence of DVT. However, the usage of anticoagulants is associated with the high risk of bleeding and wound complications [2,3]. Thus, development of a promising prevention strategy for DVT is essential.

It has been proven that bone marrow–derived circulating endothelial progenitor cells (EPCs) are recruited into resolving venous thrombi [4,5]. Our previous studies also showed that EPCs significantly improved the microenvironment and promoted the resolution of acute venous thrombus and the recanalization of chronic thrombus [6–8]. Though EPCs show splendid therapeutic potential, the clinical application of EPCs faces many challenges. Studies demonstrated that EPCs recruitment to the site of injury was limited [9,10]. Therefore, looking for a method to improve the function of EPCs is being widely discussed.

MicroRNAs are a class of ~22 nucleotides non-coding RNAs that suppress gene expression at the posttranscriptional level by promoting mRNA degradation or inhibiting mRNA translation. Recent studies have shown that microRNAs are involved in the angiogenesis process [11]. For example, miR-126, one of the most common miRNAs isolated in vascular endothelial cells, plays an important role in maintaining endothelial cell proliferation, migration and tubulogenic activity. Knock-down of miR-126 in zebrafish caused the blood vessels collapse and hemorrhage [12]. Likewise, miR-126-deficient mice exhibited the defects in the integrity of vessel, hemorrhages and partial embryos lethality [13].

MiR-150, a key regulator for the development of immune cells, was originally detected in mature lymphocyte [14]. Tano and colleagues demonstrated that ischemia mobilized bone marrow stem cells via miR-150/CXCR4 dependent mechanism [15]. Zhang et al. reported that miR-150 could be selectively packaged into microvesicles and

Abbreviations: DVT, deep venous thrombosis; PE, pulmonary embolism; EPCs, endothelial progenitor cells; PTS, post-thrombotic syndrome; EC, endothelial cells; IVC, infarenal inferior vena cava; HE, hematoxylin and eosin; MNC, mononuclear cells; AMI, acute myocardial infarction; HMEC, human microvascular endothelial cells.

* Corresponding authors at: Department of Vascular Surgery, the Second Affiliated Hospital of Soochow University, No.1055, Sanxiang Rd, Suzhou 215000, Jiangsu, China. Tel.: +86 512 67784869.
E-mail addresses: mengqy@163.com (Q. Meng), nihao229@163.com (X. Li).
[1] These authors contributed equally to this work.

0049-3848/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.thromres.2013.12.038

secreted into blood or culture medium. They found that secreted miR-150 could be delivered into HMEC-1 cells and was able to regulate the migration and vessel formation of HMEC-1 cells by targeting c-Myb [16]. However, the function of miR-150 in rat EPCs, especially in the ischemia condition such as DVT, remains unknown.

In this study, we demonstrated that ectopic expression of miR-150 enhanced rat EPCs motility and tube formation *in vitro*. To further explore the role of miR-150 in EPCs under ischemia condition, EPCs stably expressing miR-150 were achieved by lentivirus and delivered into rats with DVT. The results showed that miR-150 promoted rat EPCs homing and venous thrombus recanalization and resolution. By gain-of-function examination, c-Myb was identified as a significant target of rat miR-150, contributing to its effect. Our study revealed the function of miR-150 in EPCs *in vitro* and *in vivo* and may present a promising clinical therapy for ischemia diseases.

Materials and Methods

Animals

Male Spraque-Dawley rats were purchased from the Experiment Animal Center of Soochow University (Suzhou, China). All animal procedures were performed in accordance with institutional guidelines and approved by the University of Soochow Institutional Animal Care and Use Committee.

Isolation of EPCs

EPCs isolation, *ex vivo* expansion and culture of EPCs were performed as previous described [6–8]. Briefly, bone marrow was harvested from both femurs and tibias of SD rats. Density-gradient centrifugation with Ficoll-paque (GE Healthcare, Piscataway, NJ, USA) was used to isolate the mononuclear cells. The cells were transferred to the culture dishes pre-coated with human fibronectin (Sigma, St. Louis, MO) and then cultured in EGM-2-MV medium. On the fourth day, non-adherent cells were removed by washing with PBS. Then the adherent cells was cultured with fresh medium. On the seventh day, the EPCs were recognized as attached spindle shaped cells. The adherent cells were confluent on day 14, and passaged continuously. The second and third generation of EPCs were used for our research.

Transfection of EPCs with MicroRNA Mimics and Inhibitor

EPCs were resuspended in EGM-2-MV medium without FBS and growth factors at 5×10^7 cells/ml. A total of 120 pmol miR-150 mimics or its double-strand negative control (NC) were mixed with 100 μl EPCs and transferred to cuvettes with 0.4 cm gap. Similarly, EPCs were subjected to electroporation using 300 pmol miR-150 inhibitors or its single-strand negative control. Electroporations were performed with a Gene-Pulser II (Bio-Rad, Hercules, CA) at 180 V and 25 ms.

Scratch Wound Migration Assay

MiR-150 or NC transfected-EPCs were plated into 24-well plate with 4×10^6 cells each well. After incubation for 24 hours, linear scratch wounds were obtained by scratching the confluent cell monolayer using a 200 μl pipette tip. The medium was replaced with the serum-free medium. Images were taken at 0, 24 and 48 h and the wounding size was quantified for the cells in three wells of each group.

EPCs Migration in Transwell Chambers

A 24-well Transwell Chamber with 8.0 μm pore size (Corning Costar, Cambridge, MA) was used for migration assay. Briefly, EPCs subjected to electroporation with miR-150 or NC were plated in 6-well plate with 6×10^6 cells each well for cell viability recovery. Following incubation for 24 h, 3×10^5 EPCs suspended in 200 μl serum-free medium were seeded in the upper chamber. The bottom chamber was filled with medium containing 20% FBS. After incubation for 15 h, the non-migrating cells were removed and the transmembrane cells were dried, fixed with methanol and stained with crystal violet. The transmembrane cells were counted using a microscopy.

In vitro Angiogenesis Assay

To test whether miR-150 affected the angiogenic activity of EPCs *in vitro*, we performed matrigel tube formation assay. In brief, EPCs transfected with miR-150 mimics or inhibitor were seeded onto matrigel (BD Bioscience)-coated 48-well plate in EBM medium at a density of 5×10^4 cells per well. Following incubation for 15 h, tubular structures of EPCs in the matrigel were observed under microscope. Images of tubemorphology were taken in 5 random microscopic fields per sample at × 100 magnification. The JEDA801D pathology microscope image analysis system was applied for quantifying the cumulative mean of the tube number and length per field of view.

Vector Construction

The lentiviral expression vector pLVX-IRES-ZsGreen-miR-150 (Clontech Laboratories) was constructed to stably over-express mature sequence of miR-150 in EPCs. The 462 bp genomic segment including the mature miR-150 sequence and its 198 bp 5' and 244 bp 3'-flanking regions were amplified using primers miR-150-F/R (Table S1) and subcloned into EcoR1 and BamH1 sites of pLVX-IRES-ZsGreen vector.

Lentivirus Production and Cells Transduction

Packaging of pseudotyped recombinant lentivirus was performed by transfection of 293 T cells. Briefly, 1.6×10^6 293 T cells were plated in 6 cm dish and cultured for 20 h. Then the cells were contransfected with either 1.7 μg pLVX-IRES-ZsGreen vector or pLVX-IRES-ZsGreen-rno-miR-150, 1.13 μg pCMV Δ8.91 and 0.57 μg pMD.G using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The supernatant containing the lentivirus was harvested at 72 h and filtered through a 0.45 μm low protein binding polysulfonic filter (Millipore, Bedford, MA). EPCs, seeded in 75 cm^2 flask in advance and presenting with 10% confluence after 24 h, were infected with 1 ml lentivirus suspension in the presence of 8 μg/ml polybrene (Chemicon, Temecula, CA). After transduction for 48 h, green fluorescence was observed to indicate the transduction efficiency.

Animal Model of Venous Thrombosis and Cells Transplantation

SD rats were anethetized by intraperitoneal injection of 10% chloral hydrate. A midline laparotomy was performed. The infarenal inferior vena cava (IVC) was explored and all side branches were ligated with 7-0 Prolene suture. The posterior venous branches were blocked by electric coagulation. A 7-0 Prolene suture was tied down on the IVC just below the left renal vein. At the same time, a microvascular clamp was attached to the confluence of iliac veins for 30 min to block the blood flow and induced the thrombus in IVC. The skin was sutured and the rats were allowed to recover after the surgery. Then, the rats were divided into 3 groups for cell transplantion via tail intravenous injection: A ($n = 12$), blank control group (blank control), which received 1 ml cell culture medium; B ($n = 12$), EPCs/pLVX-IRES-ZsGreen Vector group (EPCs/vector), which received 1.0×10^6 EPCs transfected with lentivirus particle of pLVX-IRES-ZsGreen vector; C ($n = 12$), EPCs/pLVX-IRES-ZsGreen-miR-150 group (EPCs/miR-150), which received 1.0×10^6 EPCs transfected with lentivirus particle of pLVX-IRES-ZsGreen-miR-150.