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miR-24 suppression of POZ/BTB and AT-hook-containing zinc finger protein 1 (PATZ1) protects endothelial cell from diabetic damage

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ABSTRACT

The regulatory transcriptional factor PATZ1 is abnormally up-regulated in diabetic endothelial cells (ECs) where it acts as an anti-angiogenic factor via modulation of fatty acid-binding protein 4 (FABP4) signaling. The aim of the present work was to elucidate the upstream molecular events regulating PATZ1 expression in diabetic angiogenesis. The bioinformatics search for microRNAs (miRNAs) able to potentially target PATZ1 led to the identification of several miRNAs. Among them we focused on the miR-24 since the multiple targets of miR-24, which have so far been identified in beta cells, cardiomyocytes and macrophages, are all involved in diabetic complications. miR-24 expression was significantly impaired in the ECs isolated from diabetic hearts. Functionally, endothelial migration was profoundly inhibited by miR-24 suppression in Ctrl ECs, whereas miR-24 overexpression by mimics treatment effectively restored the migration rate in diabetic ECs. Mechanistically, miR-24 directly targeted the 3'untranslated region (3'UTR) of *PATZ1*, and miR-24 accumulation potentiated endothelial migration by reducing the mRNA stability of *PATZ1*. Together, these results suggest a novel mechanism regulating endothelial PATZ1 expression based on the down-regulation of miR-24 expression caused by hyperglycemia. Interfering with PATZ1 expression via miRNAs or miRNA mimics could potentially represent a new way to target endothelial PATZ1-dependent signaling of vascular dysfunction in diabetes.

1. Introduction

Vascular endothelial dysfunction predisposes diabetic patients to numerous cardiovascular complications including coronary heart disease, stroke, peripheral arterial disease and nephropathy. The diabetes-blunted angiogenesis may partially be caused by vascular endothelial growth factor (VEGF) resistance. VEGF stimulates virtually all aspects of endothelial function, namely proliferation, migration, permeability and nitric oxide production and release. Moreover, the action of VEGF makes the endothelium anti-apoptotic. In turn, the inhibition of VEGF action is associated with endothelial dysfunction. Certain signaling pathways have been so far proposed to underlie diabetic VEGF resistance during angiogenesis, including accumulation of advanced glycation end-products, reactive oxygen species (ROS) activation and inhibition of protein tyrosine phosphatases [1]. Nevertheless, the mechanisms whereby diabetes sabotages endothelial VEGF response likely involve actions at different levels and remain to be fully established.

MicroRNAs (miRNAs) are endogenous small noncoding RNA molecules that regulate a substantial fraction of the genome by

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binding to the 3'untranslated region (3'UTR) of frequently coordinately acting target messenger RNAs. Inhibition of miRNA processing by genetic knockdown of Dicer expression impairs endothelial functions and angiogenesis, indicative of a key role of miRNAs in the regulation of vascular physiology. The expression of a subset of miRNAs is closely correlated to angiogenesis [2]. miR-24 is such a striking example. miR-24 is highly conserved in various species and its expression is significantly decreased in diabetic organs and cells [3]. miR-24 acts as a critical regulator of endothelial cell apoptosis after myocardial infarction [4]. These results reveal a central role of miR-24 in vascularity.

The POZ/BTB and AT-hook-containing zinc finger protein 1 (PATZ1) is a regulatory transcription factor able to either activate or repress gene transcription depending on the cellular context. PATZ1 has been reported to play critical roles in spermatogenesis, embryonic development and DNA damage response [5]. In particular, our collaborates have recently shown that endothelial PATZ1 potently inhibits endothelial function and angiogenesis via inhibition of FABP4 expression in diabetes [6]. Therefore, the aim of our studies has been to unveil the mechanisms that could regulate PATZ1 expression in diabetic angiogenesis. To this purpose, our attention was focused on the miRNAs.

Although miR-24 is expressed in a variety of organs, it is enriched in endothelial cells [3], but its role in the vascular system, especially under certain pathologies such as diabetes, remains basically uncertain. In this study, we found miR-24 was significantly down-regulated in diabetic endothelial cells (ECs). Functionally, miR-24 could regulate endothelial migration by targeting PATZ1 signaling.

2. Materials and methods

2.1. Induction of murine diabetes

All animal procedures were approved in advance by the local ethical committee. Adult male C57BL/6 mice (about 10 weeks old), obtained from the Animal Research Center of our facility, were housed in plastic boxes individually and provided standard mouse food pellets and water *ad libitum* throughout the whole experimental period. The streptozotocin (STZ, Sigma–Aldrich, Beijing, China)-induced type 1 diabetes mellitus (DM) was induced according to the previous report [6]. Whole blood glucose obtained from the mouse tail vein was detected using a blood glucose monitor (Bioland Technology Ltd., Hong Kong, China) at the two weeks after STZ injection. Mice with blood glucose level $\geq$250 mg/dl were considered as diabetic.

2.2. Isolation, culture and treatment of heart ECs

The isolation of heart endothelial cells (ECs) from Ctrl or DM mice was carried out according to our research collaborates' work [6]. The cells were then purified by sequential affinity selection method using anti-PECAM1 antibody (BD Biosciences, San Jose, CA, USA) and anti-ICAM2 antibody (BD Biosciences). The bead-bound cells were finally recovered by a magnetic separator (Miltenyi Biotec, Shanghai, China). Cells were cultured in an endothelial basal medium (EBM-2) (Invitrogen, Shanghai, China) supplemented with 1 μg/ml hydrocortisone, 12 μg/ml bovine brain extract, 50 μg/ml gentamycine, 50 ng/ml amphothericin-B, 10 ng/ml epidermal growth factor (EGF) and 10% FCS. Isolated ECs were either immediately harvested to represent the endothelial compartment of mouse organs or cultured to passage 2 or 3 for cell migration and other assays. To investigate the effect of angiogenic stimuli, ECs were treated with VEGF (10 ng/ml, Sigma–Aldrich), sphingosine 1-phosphate (S1P, 100 nM, Tocris Bioscience, Bristol, BS, UK) or *S*-nitrosoglutathione (GSNO, 100 μM, Tocris Bioscience) for different durations, followed by Quantitative RT-PCR (RT-qPCR) analysis as described below. To manipulate the cellular levels of miR-24, cells were mixed with miR-24 mimics (10 nM) or antisense inhibitors (50 nM) (Sigma-Aldrich, Shanghai, China) in 100 μl culture medium without serum and antibiotics. Specific knockdown of PATZ1 was achieved by transfecting cells with corresponding siRNA against *PATZ1* (sc-35981) or with a control siRNA (sc-37007) (Santa Cruz Biotechnology, CA, USA). 48 h after transfection, cells were collected and subjected to other experiments.

2.3. Flow cytometry

Cytometric analyses were carried out by labeling with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human VCAM1 or CD144 (BD PharMingen, San Jose, CA, USA) at the dilution of 1:100. Flow cytometric analyses were performed using BD CellQuest acquisition and analysis software (BD FACSCalibur, CA, USA) [7].

2.4. Immunofluorescence

ECs fixed in 4% paraformaldehyde were permeabilized with 0.1% Triton X-100 for 10 min [8] and sections were incubated overnight at 4 °C with antibody against CD34 (1:100, Abcam, Hong Kong, China) or F-actin (1:100, Santa Cruz Biotechnology, Shanghai, China), followed by 1 h of incubation with Cy5/FITC-labeled anti-rabbit IgG (dilution 1:1000; Sigma). Nuclei were visualized by 10-min staining of 40,6-diamidino-2-phenylindole (dilution 1:2000; Sigma). Slides were finally analyzed by microscopy using an inverted microscope (Axio Imager M1 microscope; Zeiss).

2.5. Quantitative RT-PCR (RT-qPCR)

Total RNA was isolated from cells using RNeasy Mini Kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's protocol. For miR-24 and *PATZ1* expression analysis, total RNA was retro transcribed with microRNA-specific primers (3'-TGGCTCAGTTCAGCAGGAACAG-5') and *PATZ1* primers using TaqMan microRNA and an mRNA reverse transcription kit (Applied biosystems, Foster City, CA), respectively. Amplification of human *U6* snRNA and *18S* RNA served as internal control. The relative abundance of each target transcript was quantified using the comparative $\triangle\triangle$Ct method [9].

2.6. Cell migration assay

Confluent ECs monolayer was streaked at 1-mm width with a rubber scraper. The wound edge was marked with a coverslip placed on the dish bottom. After 36 h, the migrated cells beyond the edge were counted in 4 random fields [10].

2.7. Transwell migration assay

Confluent ECs were detached by trypsinization, suspended in DMEM containing 0.4% FBS and applied to 8-μm-pore Transwell (10^5 cells/insert) pre-coated with gelatin, which was then inserted into a well containing the same media with or without mouse VEGF (50 ng/ml; Sigma). The cells were allowed to migrate across the membrane for 6 h. The migrated cells on the lower side of the membrane were stained with DAPI, and counted in 4 random fields (20 × lens) under microscope.