FEBS Letters 587 (2013) 3792–3801

FEBS Letters

journal homepage: www.FEBSLetters.org

Dynamic expression of miR-126* and its effects on proliferation and contraction of hepatic stellate cells

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ARTICLE INFO

Article history:
Received 30 August 2013
Revised 20 September 2013
Accepted 23 September 2013
Available online 16 October 2013

Edited by Tamas Dalmay

Keywords:
Chronic liver disease
Hepatic stellate cell
miR-126*
VEGFA
Portal hypertension

ABSTRACT

In our previous study, miR-126 was identified as one of the leading miRNAs that is downregulated during activation of hepatic stellate cells (HSCs). However, the roles and related mechanisms of miR-126 in HSCs are not understood. In this study, we compared expression of miR-126 during HSC activation both in vitro and in vivo. We also applied RNA interference to analyze the role and mechanism of miR-126* in the activation of HSCs. Restoring HSCs with Lv-miR-126* resulted in decreased proliferation, accumulation of extracellular matrix components, and cell contraction, while also negatively regulating the vascular endothelial growth factor (VEGF) signal transduction pathways by partially targeted VEGF-A. Thus, we postulate that miR-126 may be a biological marker for the activation of HSCs, and useful for reducing intrahepatic vascular resistance and improving the sinusoidal microcirculation in chronic liver diseases.

1. Introduction

Accumulating evidence suggests that hepatic stellate cells (HSCs) are key players in the pathogenesis of the increased intrahepatic vascular resistance in chronic liver diseases, in which HSCs proliferate, acquire characteristics of contractile cells, and undergo trans-differentiation leading to a myofibroblast phenotype [1,2]. A majority of growth factors and related pathways are involved in the dynamic biological behavior change of HSCs. Among them, vascular endothelial growth factor (VEGF)-A is probably the most important [3]. However, full understanding of HSCs activation is still beyond our reach because of its complexity, especially the intricate regulation of gene expression.

miRNAs are several small non-coding RNAs of 21–25 nt that usually negatively modulate gene expression at the post-transcriptional level by incomplete or complete complementary binding to target sequences within the 3′untranslated region (UTR) of miRNA [4]. Previously, we reported the biological functions and targets of some miRNAs and revealed the signaling pathways regulated by these miRNAs in the activation of HSCs [5,6]. For instance, miR-15 and miR-16 regulate HSC cell growth, apoptosis and proliferation by targeting proteins involved in apoptosis and grow pathways [5,7]. These findings suggest that altered expression of miRNAs may be associated with fibrogenesis, but more studies are required to clarify the mechanism of miRNAs in the development and progression of liver diseases.

In our previous miRNA expression profiling study, miR-126 was downregulated during the activation of HSCs, and appeared to be the most enriched in quiescent stellate cells. Meanwhile, bioinformatics analysis revealed that the VEGF signaling pathway is the most enriched of these differentially upregulated signaling pathways in HSC activation. According to gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KO) analyses, we found that miR-126* is most likely related to the biological behavior of HSCs by partially targeting VEGF-related signaling pathways [5,6]. Here, we demonstrated the dynamic expression of miR-126* in chronic liver diseases, and performed an overall analysis of its effects and its related mechanisms on biological properties of HSCs, on the basis of miRNA expression profile and bioinformatics interpretation. These findings may not only increase our current knowledge about the significance of HSC biological behavior, but also provide a novel therapeutic strategy against intrahepatic vascular resistance in chronic liver diseases.

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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2013.09.047

2. Materials and methods

2.1. Isolation, culture, and identification of rat HSCs

Primary HSCs were isolated from three normal male Sprague–Dawley rats (400–500 g) by in situ perfusion and density-gradient centrifugation [8]. The rats received humane care according to the Guide for the Care and Use of Laboratory Animals of Shanghai Jiaotong University School of Medicine. All the materials for HSC isolation were obtained from commercial sources as previously described [5].

The isolated HSCs were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mmol/l glutamine. Quiescent and totally activated HSCs were harvested on days 2 and 14. Their purity was detected by immunocytochemistry for desmin (Sigma, St Louis, MO, USA) (1:100). Cell viability was determined by trypan blue staining.

2.2. Animal model of liver fibrosis and histological examination

Thirty-six Sprague–Dawley rats (250–400 g, Laboratory Animals of Shanghai Jiaotong University School of Medicine) were divided into 3 groups of 12 (normal, control, and fibrosis model). Fibrosis model rats were injected subcutaneously with 40% CCl_4 (3 ml/kg, CCl_4:olive oil, 2:3) every 3 days for 8 weeks. Control rats received only olive oil in the same way. Rats were sacrificed at 8 weeks and the degree of liver fibrosis was determined by microscopy. The intra-hepatic HSCs were isolated from CCl_4-induced fibrotic rat livers.

Liver tissues were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin, and 5-μm-thick section were prepared. All the sections were stained with hematoxylin and eosin and standard Van Gieson (VG) staining, which was used to detect collagen fibers. Total RNA from rat HSCs was prepared as described previously [6]. Fibrosis was graded according to the Ishak modified staging system [9]. Histopathology was interpreted by two independent board-certified pathologists who were blind to the study.

2.3. Immunofluorescence staining of HSCs

The expression of VEGFA in quiescent (2 days) and in culture-activated HSCs (14 days) was evaluated by immunocytochemistry. The adherent HSCs were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Sigma, St Louis, MO, USA). Following blocking in 10% preimmune goat serum for 2 h, cells were incubated with mouse monoclonal anti-VEGFA (1:100, Santa Cruz, USA) overnight at 4 °C. Then cells were incubated with TRITIC-conjugated donkey anti-mouse IgG (Sigma; 1:100) for 1 h. TRITIC fluorescence were visualized using a fluorescence microscope. The positive cells of three randomly selected areas per slide from three slides was used to calculate the expression of VEGFA in HSCs.

2.4. Double immunostaining on cryosections of rat liver

Double immunostaining on cryosections of rat liver were performed as described [10,11]. Liver tissue from five rats per group were blocked with 0.3% H_2O_2 in methanol for endogenous peroxidase activity. double staining experiments on rat livers for desmin in combination with VEGFA were performed. Immunohistochemical examination was carried out by a researcher blind to the experimental design. The percentage of cells co-expressing VEGF/desmin was determined by counting the number of VEGF-positive cells in desmin-positive cells in three different fields per slide from three slides.

2.5. Lentiviral construction, production and transfection

The precursor miR-126* (pre-miR-126) sequences were obtained from miRBase (http://microrna.sanger.ac.uk/sequences/). The premiR-126 sense and antisense primers were: 5′-GCGAATTC-CAGAGGGCAGCTAGCCCT-3′, 5′- GCGGATCCAAGCCTCACCTGTTCT-3′. Lentivector Expression System was purchased from System Biosciences. Packaging and production of lentivirus were performed according to the manufacturer's protocol. Briefly, we first extracted DNA, then designed PCR primers. The product was amplified by PCR contains a sequence of miRNA precursor, which was confirmed by enzyme digestion and then cloned into into the pCDH-CMV-MCS-EF1-copGFP miRNA expression vector (System Biosciences). The new miRNA expression vectors (pCDH-CMV-MCS-EF1-copGFP-miR-126) and Lentivirus Package plasmid mix (System Biosciences) were cotransfected into 293TN cells with Lipofectamine 2000 (Invitrogen). The culture supernatants were collected, concentrated, and used as a virus stock. The viral titer was determined by counting green fluorescent protein (GFP)-positive cells after transfection.

Primary myofibroblast-like HSCs, which had experienced total activation at day 14 and then divided into a blank group, Lv-GFP group, and Lv-miR-126* group with a multiplicity of infection (MOI) from 10 to 50. For 24 h prior to infection, HSCs were plated in each well of 6-well plates, then infected with recombinant viruses (Lv-miR-126* or LV-GFP) at different MOI of for 6 h at 37 °C, followed by the addition of fresh growth medium. Three days later, all lentiviral vectors expressed enhanced GFP, which allowed for titration and measurement of their infection efficiency in transfected cells.

2.6. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of miR-126* and VEGF expression

HSCs from 3 rats per group were isolated from rat livers by perfusion of collagenase and pronase, followed by centrifugation over Nycodenz gradient, as described above. RNA purity and concentration were determined by electrophoresis and a BioPhotometer (Eppendorf AG, Hamburg, Germany). The extracted total RNA of HSCs from the three groups was reverse transcribed into cDNA using ExScriptTM RT reagent Kit (TAKARA, Kusatsu, Japan). Expression of mature miRNA was assayed using stem-loop RT followed by PCR analysis, as previously described [5]. PCR analysis was performed in triplicate for each sample. The relative amount of miRNA was normalized against U6 snRNA and VEGF was normalized against GAPDH, and the fold change was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences were listed in Table 1.

2.7. Detection of VEGF-A pathway by Western blotting

Total proteins were prepared by standard procedures and quantified by the BCA method (Pierce, Rockford, IL, USA). Thirty micrograms of protein per sample were loaded onto a 10% SDS–polyacrylamide gel. After electrophoresis, the protein was transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) by electro-elution. The membrane was incubated with anti-VEGF-A (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) / phosphoinositide 3-kinase (PI3K; 1:200; Santa Cruz Biotechnology)/AKT (1:200; Santa Cruz Biotechnology)/ CCND1 (1:100; Santa Cruz Biotechnology) antibody overnight at 4 °C and with horseradish-peroxidase-conjugated goat anti-mouse IgG (1:2000; Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at room temperature. After washing, the membrane was