Experimental and Molecular Pathology 91 (2011) 471–477

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

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

MicroRNA-126 inhibits ischemia-induced retinal neovascularization via regulating angiogenic growth factors

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ARTICLE INFO

Article history:
Received 21 September 2010
and in revised form 21 April 2011
Available online 6 May 2011

Keywords:
MicroRNA
Retinal neovascularization
Oxygen-induced retinopathy
VEGF
IGF-2
HIF-1α
p38
ERK

ABSTRACT

To investigate the potential transcriptional regulation and signal pathway of a single microRNA in ischemia-induced retinal neovascularization (NV), we used oxygen-induced retinopathy (OIR) in establishing retinal NV model, and quantitative real-time reverse transcriptase PCR analyzing a microRNA (miR-126) alteration. The mice were treated with plasmid pCMV-MIR-126/liposome mixture intravitreal injection, using pCMV-MIR/liposome mixture as control. The expression levels of VEGF, IGF-2 and HIF-1α, and the level changes of total and phosphorylated p38, ERK in retina from OIR mice were determined by western blot analysis. The effects of miR-126 on retinal NV in OIR mice were identified with fluoresecin angiography and H & E staining. No effect of miR-126 intravitreal injection on retinal vessels was performed with CD31 stained retinal sections. Our results showed that miR-126 was significantly decreased in retina from OIR mice. We confirmed that restoration of miR-126 in retina overcame the high levels of VEGF, IGF-2 and HIF-1α through downregulating p38 and ERK signaling molecules in OIR model, and that miR-126 intravitreal injection reduced retinal NV in OIR model. These results suggest that miR-126 might play a potential transcriptional role in the pathogenesis in diabetic retinopathy.

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Introduction

Retinal neovascularization (NV) is one of the major features of several eye diseases including diabetic retinopathy (DR), which are mediated by angiogenic growth factors, such as vascular endothelial growth factor (VEGF), insulin/insulin-like growth factor (IGF) (Lofqvist et al., 2009) and hypoxia-inducible factor-1 alpha (HIF-1α) (Aiello et al., 1994; Miller et al., 1997; Xiao et al., 2003). The pathological mechanism of retinal NV has not yet been clearly elucidated. Understanding the transcriptional regulations on these factors might explore the role of transcriptional regulators in vascular pathological changes in eyes.

Over the past few years, microRNAs (miRNA) have emerged as a prominent class of gene regulators. miRNAs were identified in many sorts of mammalian tissues and organs including eyes. It is increasing that miRNAs are important in regulating these factors in diseases. Some publications show that signaling of VEGF (Nicoli et al., 2010), IGF-2 (Polesskaya et al., 2007) and HIF-1α (Yamakuchi et al., 2010), which are crucial for angiogenesis in many diseases, could be regulated by microRNAs. Shen et al. reported that microRNAs could regulate ocular NV (Shen et al., 2008). However, it has not been shown whether a key miRNA plays a crucial role in the retinal NV. It is supposed that some miRNA might be crucial for the regulation in this pathological process.

miRNAs are small noncoding RNAs on average only 22 nucleotides, that are thought to regulate gene expression through sequence-specific base pairing with target mRNAs. It has been reported that microRNAs act as critical transcriptional factors on regulating their target genes. Over 1000 microRNAs have been identified using molecular cloning and bioinformatics prediction strategies (Bentwich et al., 2005; Chen, 2005). Most microRNAs are thought to function through the inhibition of effective mRNA translation of target genes through imperfect base pairing with the 3′-untranslated region (3′-UTR) of target mRNAs (Doench and Sharp, 2004). There is increasing evidence suggesting that miRNAs play critical roles in many key processes, such as vascular angiogenesis (Kuhnert et al., 2008; Chen and Gorski, 2008; Kuehbacher et al., 2007), cell growth, tissue differentiation, cell proliferation (Johnson et al., 2007), embryonic development, and apoptosis. miRNAs also play important roles in cellular signaling network (Cui et al., 2006), cross-species gene expression variation (Cui et al., 2007a, 2007b), and co-regulation with transcription factors (Cui et al., 2007a, 2007b). As such, reduction of miRNAs, dysfunction of miRNA and dysregulation of miRNAs and their targets may result in disorders. However, it is still unclear whether the transcriptional regulation of microRNAs is implicated in the retinal NV in many eye diseases. And understanding

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0014-4800/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexmp.2011.04.016

the related mechanism is crucial for the design of therapeutic strategies to the diseases.

Some microRNAs, such as miR-31, miR-150 and miR-184, were identified to reduce in OIR mouse model as reported by Shen et al. To our interesting, miR-126 is regarded as one of the most important microRNAs governing vascular integrity and angiogenesis via regulating the signaling of angiogenic growth factors, such as VEGF and FGF (Fish et al., 2008; Wang et al., 2008). It might be crucial for retinal NV in eye diseases. Although there is no evidence to show miR-126 reduced in OIR model, our preliminary data supported that miR-126 was a candidate microRNA chosen to treat this study. In this study, we generated oxygen-induced retinopathy (OIR) mouse model and demonstrated that miR-126 was significantly decreased in retina from OIR mice. We also showed the effects of restoration of decreased miR-126 on ischemia retinal vascular morphological changes by regulating the expression levels of angiogenic factors, and possible signal pathway in retina from OIR mice. It might suggest the important regulation by miR-126 in retinal NV of DR.

Materials and methods

Establishment of oxygen-induced retinopathy mouse model

All animal experiments were conducted in accordance with the guidelines of the ARVO statement for the "Use of Animals in Ophthalmic and Vision Research" and were approved by Institutional Animal Care and Use Committees. In the neonatal mouse model of oxygen-induced retinopathy, 7-day-old C57BL/6 mice were kept with their nursing dams in a 75% oxygen chamber for 5 days until postnatal day (P)12. Immediately after exposure, they were transferred to room air until P17.

Quantitative real-time reverse transcription PCR assay for miR-126 expression

To ensure the significant reduction of miR-126 according to our previous result of microarray analysis, we performed quantitative real-time reverse transcription-PCR assay for miR-126 expression in retina from the eyes of OIR mice. The mouse pups were sacrificed on P12, and the retina were removed. Using a small RNA isolation kit (Ambion), total RNA and miRNA fractions were isolated from retina of OIR mice and the age-matched normal mice. miRNA expression levels were quantified using *mir*Vana qRT-PCR miRNA detection protocol (Ambion). 5S-rRNA was used as a control. Each PCR reaction was carried out in triplicate in a 25 μl volume using SYBR Green Assay Master Mix (Applied Biosystems) for 3 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 45 s in a Bio-Rad I Cycler (Bio-Rad Laboratories). miRNA levels were quantified based on the ratio of miRNA/5S-rRNA using the formula, $2^{(\mathrm{CTmicroRNA-CT5S})}$.

Plasmids preparation, identification and purification

The plasmid vector pCMV-MIR (Origene) with green fluorescein protein(GFP) report gene was used to construct the pCMV-MIR-126 plasmid. The sequence of resulted vector was verified by sequence analysis (Origene). The blank plasmid vector pCMV-MIR was used as a control. Plasmids pCMV-MIR and pCMV-MIR-126 were identified with digestion by SgfI and MluI restriction enzymes. Using 0.8% agarose gel electrophoresis, the DNA bands were observed under the UV light. Plasmids pCMV-MIR and pCMV-MIR-126 were transformed in competent cell DH5α and extracted with Qiagen Plasmid Midi Kit (Qiagen).

Plasmids/liposome mixture intravitreal injection

Plasmids pCMV-MIR or pCMV-MIR-126, were packaged into Lipofectamine™ 2000 Transfection Reagent (Invitrogen) in accordance with the manufacturer's instructions, and suspended in HEPES buffer separately. OIR mice and normal control mice on P12 were anesthetized by intraperitoneal injection of ketamine hydrochloride (40 mg/kg) and xylazine hydrochloride (10 mg/mL), and then received an intravitreous injections of 2.5 μg of pCMV-MIR-126 in one eye and 2.5 μg of pCMV-MIR in the other eye of each animal. Intravitreal injection was performed by inserting a 32-gauge Hamilton needle 200 μm into the vitreous of anesthetized OIR mice at a site 1-mm posterior to the limbus of the eye. Insertion and infusion were performed and directly viewed through an operating microscope. Care was taken not to injure the lens or the retina. The tip of the needle was positioned over the optic disk and a 5-μl volume was slowly injected into the vitreous. Any eyes that exhibited damage to the lens or retina were discarded and not used for analyses (Poulaki et al., 2004).

To identify the location of injected plasmids in eyes, the frozen eyeballs from OIR mice on P17 with intravitreal injection were cross-sectioned vertically through the center of the cornea and optic nerve. Then the green fluoresecent protein (GFP) signal was examined through fluorescent microscopy. The nuclei were counterstained with PI (red).

Western blotting analysis

OIR mice were sacrificed on P17 after 5 days with plasmids/liposome mixture intravetrial injection treatment. The eyeballs were dissected, retina and vitreous were collected and homogenized in 150 μl ice-cold tissue lysis buffer (50 mmol/l Tris–HCl, pH 7.4, 2 mmol/l $MgCl_2$, 1% NP-40, 10% glycerol, 100 mmol/l NaCl) containing a protease inhibitor cocktail (1:100 dilution, Sigma Aldrich). The lysates were cleared by centrifugation at 12,000 *g* for 20 min at 4 °C. The protein concentration of the lysate was determined using the Bradford assay. The same amount of proteins (20 μg) were resolved by SDS-PAGE in 10% denaturing gels and transferred onto nitrocellulose membranes (Bio-Rad). Immunoblotting was performed by incubating the membranes with the primary antibodies against rabbit anti-VEGF (1:1000 in 5% nonfat milk powder/0.1% PBS Tween-20; Santa Cruz), IGF-2 (1:1000; BD Transduction Laboratories), p38 (1:1000; Cell Sigaling), phospho p38 (1:1000; Cell Sigaling), ERK (1:1000; Cell Signaling), phospho ERK (1:1000; Cell Signaling) or β-actin (1:2000; Abcam) antibodies overnight at 4 °C. After incubating with secondary IgG conjugated horseradish peroxidase (Santa Cruz), the membranes were developed with chemiluminescence. The signal intensity was quantified by densitometry using software.

Fluorescein angiography of retinal neovascularization

C57BL/6 mouse pups subjected to plasmids/liposome mixture intravetrial injection treatment were anesthetized on P17. Retinal vasculature was visualized by fluorescein angiography as previous report (Bai et al., 2009). Mice were perfused through the left ventricle with 10 mg/ml high-molecular weight fluorescein–dextran (Sigma) in PBS. Eyes were enucleated and fixed in darkness with 4% cold paraformaldehyde for 2 h. After removal of the lens, the retina was flat-mounted with glycerol–gelatin. The vasculature was analyzed with fluorescent microscopy. The neovascular area was calculated using Photoshop 8.0 software. With its software freehand tool, neovascularization was quantified by comparing the number of pixels in neovascular area with the total number of pixels in the whole retina. To avoid 'bias', quantification was performed in a 'blind' fashion.

Retinal vascular endothelial cell proliferation

After OIR mice were sacrificed on P17, the eyeballs were obtained and the lenses were removed. Then the eyeballs were fixed at 25 °C for 48 h in Perfix (4% paraformaldehyde, 20% isopropanol, 2% trichloroacetic acid, 2% zinc chloride) and soaked in 70% Ethanol for

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