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miR-146a suppresses STAT3/VEGF pathways and reduces apoptosis through IL-6 signaling in primary human retinal microvascular endothelial cells in high glucose conditions

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ABSTRACT

microRNA (miRNA) play critical roles in the pathological processes of diabetic retinopathy, including inflammatory responses, insulin signaling, and angiogenesis.

In addition to their regulatory functions on gene expression, miRNA is considered as a potential therapeutic target, as well as a diagnostic marker for many diseases. Our understanding on the pathological mechanisms underlying diabetic retinopathy is still incomplete and additional investigations are required to develop novel therapeutic strategies. The aim of this study was to investigate our hypothesis that miR-146a plays a role in suppressing pro-inflammatory pathways, involving STAT3 and VEGF, through regulating IL-6 signaling to reduce apoptosis of human retinal endothelial cells (REC) in high glucose conditions. Human REC were cultured in normal (5 mM) glucose or high glucose medium (25 mM) for 3 days. We performed transfections on REC with miRNA mimics (hsa-miR-146a-5p). Overexpression of miR-146a reduced IL-6 levels, STAT3 phosphorylation, and VEGF levels in REC cultured in high glucose. Cellular apoptosis was decreased in REC overexpressing miR-146a, as demonstrated by the inhibition of DNA fragmentation. More importantly, we demonstrated that the regulatory role of miR-146a on STAT3/VEGF and apoptosis was mediated by IL-6 receptor signaling in REC.

Overall, we report that miR-146a suppressed IL-6 signaling, leading to reduced levels of STAT3 and VEGF in REC in high glucose conditions, leading to decreased apoptosis. The outcome suggests that miR-146a is a potential molecular target for inhibiting inflammation and apoptosis in the diabetic retina through the suppression of the IL-6-mediated STAT3/VEGF pathway.

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1. Introduction

In 2012, 9.3% of Americans or 29 million people had diabetes (National Diabetes Statistics Report, 2014). The prevalence of diabetes will increase by 366 million people worldwide by 2030; with the population at risk of blindness escalating with increased diabetes incidence, according to World Health Organization (WHO).

Abbreviations: miR, microRNA; REC, retinal endothelial cells; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; IL-6, interleukin-6; sIL-6R, soluble interleukin-6 receptor; mIL-6R, membrane-bound interleukin-6 receptor; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor; ADAM-10 and -17, a disintegrin and metalloproteinase domain-10, and -17.

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The molecular mechanisms of diabetic retinopathy remain poorly understood with extensive investigations ongoing focusing on the cellular pathways underlying hyperglycemic-induced retinal damage and diabetic retinopathy, with the goal of development of novel strategies for therapeutics. miRNA regulate target genes by binding to 3' UTR of mRNA, inducing post-transcriptional reduction in gene expression (Olena & Patton, 2010). miRNA can be detected in serum, plasma, and a variety of body fluids, including saliva and urine (Cortez & Calin, 2009; Igaz & Igaz, 2015). Thus, miRNA are novel and powerful candidates for diagnostic biomarkers and for assisting in the development of therapeutic strategies (McClelland & Kantharidis, 2014). Altered expressions of miRNA have been reported in a variety of pathological conditions, including diabetic retinopathy (Sun et al., 2013; Xiong et al., 2014) and inflammatory responses (Ichii et al., 2012; Sonkoly, Stahle, & Pivarcsi, 2008).

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There is increasing evidence of the functions of miRNA in many diseases, we still have a limited understanding on how specific miRNA may influence the molecular mechanisms in the pathology of diabetic retinopathy. miR-146a is one of many candidates, in addition to miR-15b-, -16, -18b, -29, -195, and -200b, -221, that have been studied for a regulatory role in diabetic retinopathy and/or hyperglycemia (Cao, Feng, Chen, Chu, & Chakrabarti, 2014; Cowan et al., 2014; Fulzele et al., 2015; Li et al., 2009; Lin et al., 2016; Mortuza, Feng, & Chakrabarti, 2014; Wu et al., 2016; Ye & Steinle, 2015). In addition to diabetic retinopathy, miR-146a has been studied in different types of ocular disorders, such as corneal disease (Funari et al., 2013), age-related macular degeneration (Menard et al., 2016), uveal melanomas (Ragusa et al., 2013), autoimmune uveo-retinitis (Watanabe, Keino, Kudo, Sato, & Okada, 2016), and Graves' ophthalmopathy (Li, Du, Jiang, & He, 2014). In diabetic retinopathy, reduced levels of miR-146a were found in the serum of T2D patients, which was associated with chronic inflammation (Baldeon et al., 2014). Also, modified rhythmic expression of miR-146a was shown in human retinal endothelial cells from diabetic donors (Wang et al., 2014). Our previous study demonstrated inhibitory roles of miR-146a on high glucose-induced TLR4 signaling leading to altered NF- κ B and TNF α in REC (Ye & Steinle, 2016).

Elevated IL-6 levels, a key pro-inflammatory cytokine, have been reported in diabetic retinopathy (Canataroglu et al., 2005; Kawashima, Shoji, Nakajima, Kamura, & Sato, 2007; Koskela, Kuusisto, Nissinen, Savolainen, & Liinamaa, 2013; Yoshida et al., 2015). IL-6 was reported as a potential target of miR-146a, as shown by the homology between the 3'-UTR of human IL-6 mRNA and miR-146a (Wu et al., 2013). Negative regulatory effects of miR-146a on IL-6 were reported in patients with T2DM (Balasubramanyam et al., 2011) and in human aortic endothelial cells (Wu et al., 2013). Classic IL-6 signaling works through membrane bound IL-6 receptors, which is found on only limited types of cells, including subsets of leukocytes, epithelial cells, and hepatocytes (Rose-John, 2012). It is suggested that IL-6 *trans*-signaling, acting through soluble IL-6 receptor (sIL-6R), plays a role in the pathophysiology of diabetic retinopathy. Elevated levels of IL-6 and sIL-6R were shown in the serum and aqueous humor of subjects with diabetic retinopathy (Chen, Zhang, Liao, & Wen, 2016). In addition, higher levels of sIL-6R were found in the serum and vitreous fluid of patients with proliferative DR. Furthermore, the levels of sIL-6R had a significant correlation with VEGF levels in the vitreous fluid (Kawashima, Shoji, Nakajima, Kamura, & Sato, 2007).

IL-6 can stimulate Jak/STAT3 signaling in the eye (Elsaedi, Bembem, Zhao, & Goldman, 2014; Fasler-Kan et al., 2013; Samardzija et al., 2006). Additionally, high glucose can stimulate STAT3 phosphorylation in retinal endothelial and RPE cells (Chen et al., 2012; Li et al., 2012; Zheng et al., 2010). The activation of STAT3 plays a role in inflammatory pathways and can initiate ER stress under high glucose conditions in TR-iBRB cells (Chen et al., 2012). Moreover, IL-6-induced STAT3-mediated nitric oxide resulted in apoptosis (Oh, Lee, Park, & Jun, 2011). Phosphorylation of STAT3 played a mediatory role in retinal neuronal apoptosis in streptozotocin (STZ)-diabetic rats Li et al., 2011. Additionally, STAT3-induced apoptosis was mediated through the downregulation of PI3K-Akt pathway in mammary epithelial cells (Abell et al., 2005) and through Bcl-2/Fas signaling in cerebral ischemia/reperfusion (Guo, Yin, Zi, & Yan, 2016).

Vascular endothelial growth factor (VEGF), one downstream effector of the STAT3 pathway, is a crucial factor in diabetic retinopathy, mediating increased vascular permeability and pathological angiogenesis (Witmer, Vrensen, Van Noorden, & Schlingemann, 2003). Upregulation of VEGF mediated by STAT3 has been shown in bovine retinal capillary endothelial cells under

high glucose conditions (Zheng et al., 2010). In addition, the proliferation of endothelial cells induced by VEGF was inhibited when STAT3 was reduced in HUVEC and human retinal microvascular endothelial cells (Wang et al., 2014). A stimulatory role of VEGF on cell death was shown in mouse cerebral endothelial cells after oxygen-glucose deprivation (Narasimhan, Liu, Song, Massengale, & Chan, 2009).

Understanding cell type- and disease-specific functions of miR-146a is crucial for development of potential therapeutics for clinical application in diabetic retinopathy. In the present study, we tested the hypothesis that elevated levels of miR-146a suppress IL-6 signaling to inhibit pro-inflammatory pathways of STAT3 and VEGF, leading to the prevention of REC apoptosis in high glucose conditions.

2. Materials and methods

2.1. Cell culture

Human REC were cultured as described in our previous study (Ye & Steinle, 2015). Briefly, REC were purchased from Cell Systems Corporation (CSC, Kirkland, WA). Cells are grown in Cell Systems Medium. Microvascular growth supplement (MVGS, Invitrogen), 10 μ g/mL gentamycin, and 0.25 μ g/mL amphotericin B are added to all cell cultures. Cells are maintained in normal (5 mM) glucose or transferred to high glucose medium (25 mM) (Cell Systems) for 3 days. Only primary cells within passage 5 were used. Cells were quiesced by incubating in the appropriate medium without growth supplementation for 20 h before the experiments.

2.2. Cell transfection with microRNA-mimics and IL-6 siRNA

REC transfection was performed using the miRNA mimic (hsa-miR-146a-5p) (Invitrogen, Carlsbad, CA) or IL-6 siRNA (Dharmacon, Lafayette, CO) using oligofectamine (Invitrogen), following manufacturer instructions. We transfected cells for 48 h before the cell harvest. A final concentration of 50 nM was used for the mimic, with a negative control for all studies with equal concentration of the mimic negative control (Invitrogen) in REC grown in high glucose. For studies with human IL-6 siRNA, a final concentration of 20 nM was used and a negative control group was used for all studies using an equal concentration of a scrambled siRNA (Dharmacon, Lafayette, CO) in REC grown in high glucose. Mock-treated controls, normal glucose (NG) and high glucose (HG), were treated with oligofectamine only.

2.3. Real-time quantitative PCR

Total RNA was isolated and purified using the Trizol method. Purity and quantity of RNA was measured using a Synergy HTX multi-mode reader (BioTek; Winooski, VT). For polyA tail reverse-transcriptase PCR, 5 μ g of total RNA was treated with DNase I for 15 min at room temperature (Promega; Madison, WI) followed by the addition of polyA using (polyA) polymerase (NEB; Ipswich, MA) at 37 $^{\circ}$ C for 1 h. The final reaction mixtures were extracted with phenol/chloroform, precipitated with isopropanol, and re-dissolved in 25 μ l diethylpyrocarbonate (DEPC)-treated water. PolyA-tailed RNA (6 μ l) was reverse-transcribed into first-strand cDNA using Superscript II reverse transcriptase (Invitrogen) with the oligo-dT adapter primer 5'GC GAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTVN3'. For PCR, 1 μ l of RT product was diluted three times and used as a template in each reaction. Sequences of primer pairs used to detect human IL-6R (Life Technologies, Carlsbad, CA) were as follows (47): 1) mL-6R, fwd: 5'-CTCCTCTGCATTGCCATTGT-3', rev: 5'-TGT

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