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Rg1 inhibits high glucose-induced mesenchymal activation and fibrosis via regulating miR-2113/RP11-982M15.8/Zeb1 pathway

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ABSTRACT

Recent study has showed that Ginsenoside Rg1, the mian active compound of Panax ginseng, could ameliorate oxidative stress and myocardial apoptosis in diabetes mellitus. However, the roles and mechanisms of Rg1 in proliferative diabetic retinopathy (PDR) are still unclear. In the present study, we aimed to investigate the effects of Rg1 on mesenchymal activation of high-glucose (HG) cultured müller cells. High glucose conditions up-regulate MMP-2, MMP-9 and down-regulate TIMP-2, and promote mesenchymal activation in Müller cells. And Rg1 inhibits the HG-induced mesenchymal activation and HG-increased MMP-2 and MMP-9 and HG-decreased TIMP-2 in Müller cells. HG up-regulates Zeb1 and lncRNA RP11-982M15.8, and down-regulates miR-2113, and Rg1 inhibits these effects of HG. Both inhibition of miR-2113 and over-expression of RP11-982M15.8 significantly restored the HG induced mesenchymal activasion. Taken together, our findings suggested that Rg1 inhibited HG-induced mesenchymal activation and fibrosis via regulating miR-2113/RP11-982M15.8/Zeb1 pathway.

1. Introduction

The estimated number of diabetes mellitus patients was 382 million in 2013, and this number was expected to rise to 592 million by 2035 on a global scale [1]. More than 60% of the people with diabetes live in Asia, with almost one-half in China and India combined [2]. Diabetic retinopathy (DR) is a common and severe microvascular complication of diabetes [3]. DR is commonly divided into the early stage non-proliferative DR (NPDR) and the advanced stage proliferative DR (PDR). Although the treatments for PDR have greatly improved over the past decade, PDR remains the leading cause of blindness among working individuals in developed countries [4]. Hence, it's necessary to explore a novel pharmacological target to manage PDR.

Müller cells are the major glial cells in the retina and participated in the pathogenesis of diabetic retinopathy, and many studies showed that müller cells are expressed in the diabetic epiretinal membrane [5]. Müller cells produced and secreted cytokines and growth factors which could cause retinal neuron and capillary cell dysregulation [6]. Previous study showed that in high glucose treated retinal müller cells and diabetic humans, the activity of caspase-1 was increased [7]. Müller cells had the characteristics of fibroblast cells, and high expression of mesenchymal markers such as vimentin was correlated with reduced renal function in diabetic patients [8]. Zhou et al. further that high glucose exposure could increase the mesenchymal marker expression in müller cells [9], however, the underlying mechanisms and how to inhibit the mesenchymal activation were still largely unknown.

Ginsenoside (Rg1) is one of the active ingredients in panax ginsen. In the studies of diabetes, Rg1 functions as the protecting molecules against injuries. After Rg1 treatment in diabetic rats, myocardial apoptosis was inhibited and caspase-3 expression was down-regulated [10]. Tian et al. found that Rg1 inhibited hepatocellular apoptosis by down-regulating the protein levels of active caspase-3 and BAX, and lowered the blood glucose level and insulin resistance index in T2-DM rats [11]. Yu et al. found that Rg1

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appeared to ameliorate diabetic cardiomyopathy by inhibiting ER stress-induced apoptosis in diabetic rats [12]. Whether Rg1 could regulate the mesenchymal activation was still unknown.

In the present study, we revealed that Rg1 inhibited HG-induced mesenchymal activation via regulating miR-2113/RP11-982M15.8/Zeb1 axis in Müller cells.

2. Materials and methods

2.1. Cell culture

Human Müller cells (MIO-M1) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, USA), and maintained at 37 °C in a humidified atmosphere with 5% CO2. Cells were incubated with normal D-glucose (5.5 mM) or high D-glucose (25 mM) for 48 h.

2.2. Total RNA extraction and real-time PCR assay

Total RNA was extracted from the cells with RNAiso plus (Takara, Japan). Total RNA (2 μg) was reverse-transcribed into cDNA with random hexamer primers (Takara, Japan). Real-time PCR was used to detect the mRNA expression levels of Zeb1, RP11-982M15.8 and GAPDH. The PCR reactions were performed in a total volume of 20 μl, including 10 μl of 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, USA) and 1 μl of primer mix (10 μM). All PCR experiments were performed using a LightCycler (Roche, Germany) as follows: an initial denaturation at 95 °C for 10 min; 45 cycles at 95 °C for 15 s and 60 °C for 1 min. The relative gene expression was calculated using the comparative CT method. The gene expression of target gene was normalized to an endogenous reference (GAPDH), and relative to the calibrator were given by the formula $2^{-\Delta\Delta Ct}$. ΔCt was calculated by subtracting the average GAPDH Ct from the average Ct of the gene of interest. The primers were as follows: Zeb1 forward primer, 5′- GACTCTGATTCTACACCGCCC-3'; Zeb1 reverse primer, 5′- AGCGCTTTCCACATTTGTCAC-3'; RP11-982M15.8 forward primer, 5′-GAAACCTCAAGGGGCAGTACA-3'; RP11-982M15.8 reverse primer, 5′-TTGGGAATGTCATGGCTCCTC-3'; GAPDH forward primer, 5′-AAATCCCATCACCATCTTCCAG-3'; GAPDH reverse primer, 5′-GAGTCCTTCCACGATACCAAAGTTG-3'.

2.3. Western blot assay

Cells were lysed in the lysis buffer (20 mM Tris, 2 mM EDTA, 50 mM 2-mecaptoethanol, 10% glycerol, pH 7.4). Cell lysates were placed on ice for 30 min and centrifuged at 12,000 g for 15 min at 4 °C. Afterward, the protein samples were separated by SDS-PAGE, and then transferred to polyvinylidene difluoride membrane. The membranes were blocked with 10% non-fat milk for 2 h at room temperature and then incubated with specific primary antibodies overnight at 4 °C and probed with corresponding secondary antibodies for 1 h at room temperature. Detection was done by chemiluminescence (Milipore Corporation, USA).

2.4. Immunouorescent staining

Immunouorescent staining was performed as described previously [13]. Vimentin (5741, cell signaling technology) primary antibody was used at dilutions of 1:50. The cells were incubated with Vimentin primary antibody overnight at 4 °C, followed by incubation with Alexa Flour 594 goat anti-rabbit secondary antibody at dilutions of 1:200 for 1 h. Then the nuclei of cells were stained with DAPI.

2.5. Statistical analysis

The data were analyzed by student's t-test using the Graphpad Prism 5.0. $P < 0.05$ was considered to indicate a statistical significant difference.

3. Results

High glucose conditions up-regulate MMP-2, MMP-9 and down-regulate TIMP-2, and promote mesenchymal activation in Müller cells.

We cultured the Müller cells on the conditions of 30 mM high glucose for 48 h, and found that MMP-2 and MMP-9 were significantly increased and TIMP-2 was significantly decreased using Western blotting assay (Fig. 1A). We also examined the protein levels of mesenchymal markers in the Müller cells exposed to high glucose conditions. And the results showed that the expressions of α-SMA, N-cadherin and Vimentin were significantly up-regulated in high glucose treated cells compared with control cells (Fig. 1B). Using immunofluorescent staining method, we confirmed that high glucose significantly increased the Vimentin protein level (Fig. 2A).

Rg1 inhibits the HG-induced mesenchymal activation and HG-increased MMP-2 and MMP-9 and HG-decreased TIMP-2 in Müller cells.

In our experiment, Rg1 significantly inhibited the HG-increased Vimentin level in the immunofluorescent staining assay (Fig. 2B). We further found that when we treated the HG cultured Müller cells using Rg1, the MMP-2 and MMP-9 were decreased, and TIMP-2 was increased compared with HG group (Fig. 2C). Rg1 treatment also inhibited the HG-induced α-SMA, N-cadherin and Vimentin expressions compared with HG group in western blotting assay (Fig. 2D).

HG up-regulates Zeb1 and lncRNA RP11-982M15.8, and down-regulates miR-2113, and Rg1 inhibits these effects of HG.

Zeb1 is the important transcription factor in EMT process. And we found that HG significantly up-regulated the protein and mRNA expressions of Zeb1, and Rg1 significantly inhibited HG-induced

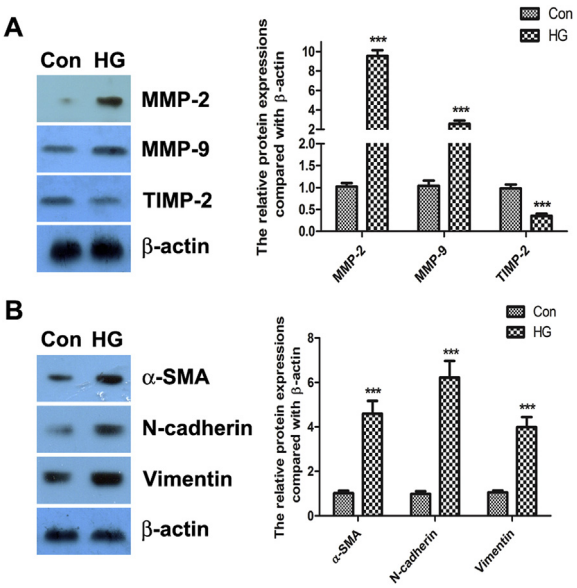

Fig. 1. High glucose conditions up-regulate MMP-2, MMP-9 and down-regulate TIMP-2, and promote mesenchymal activation in Müller cells. A. Western blotting assay was used to detect the expressions of MMP2, MMP9 and TIMP-2. B. Western blotting assay was used to detect the expressions of α-SMA, N-cadherin and Vimentin. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.