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HMGB-1 as a Potential Target for the Treatment of Diabetic Retinopathy

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Diabetic retinopathy (DR) is one of the most important complications of diabetes mellitus (DM) and is the leading cause of blindness in diabetic patients. Recent studies showed that as important inflammatory mediators, high mobility group box 1 (HMGB-1) is associated with diabetic peripheral neuropathy and can participate in the occurrence and development of DR. This study explored HMGB-1 as a therapeutic target for DR treatment through observing its role in retinal ganglion cells (GRCs) in a high glucose environment.

Material/Methods: RGCs were randomly divided into 3 groups: the normal control group, the high glucose group, and the siRNA HMGB-1 group. Real-time PCR was used to detect HMGB-1 mRNA expression. ELISA was used to test HMGB-1 protein expression in the supernatant. MTT assay was performed to determine cell proliferation. Real-time PCR and Western blotting were used to analyze TLR4 and NF-κB expression.

Results: HMGB-1 mRNA was up-regulated (P=0.015) and protein secretion increased (P=0.022) in the high glucose environment. RGCs survival decreased (P=0.026), while TLR4 and NF-κB mRNA (P=0.009 and P=0.017, respectively) and protein expression increased significantly (P=0.041 and P=0.024, respectively). SiRNA HMGB-1 transfection obviously inhibited HMGB-1 mRNA expression (P=0.032), reduced HMGB-1 secretion (P=0.012), and decreased TLR4 and NF-κB mRNA (P=0.033 and P=0.024, respectively) and protein expression (P=0.032; P=0.027, respectively). Compared with the high glucose group, the RGCs survival rate increased significantly (P=0.037).

Conclusions: As a therapeutic target, HMGB-1 can inhibit inflammation and promote RGCs survival to delay DR progress through the HMGB-1-TLR4-NF-κB signaling pathway.

MeSH Keywords: Diabetic Retinopathy • Retinal Ganglion Cells • Toll-Like Receptor 4

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Background

Diabetic retinopathy (DR) is one of the main complications of diabetes mellitus (DM), and is also the leading cause of blindness in diabetic patients [1,2]. According to a WHO report, there are currently 360 million people world-wide with DM, and this number will reach 1 billion in 2030 based on the current prevalence rate [3,4]. It was found that retinal nerve cell damage in retinopathy occurred far earlier than the microvascular lesions. Many patients without retinal microvascular lesions exhibited visual function decline, including abnormal electroretinogram (ERG), reduced dark adaptation ability, and visual field damage [5,6]. As the earliest-differentiated nerve cells in the retina, retinal ganglion cells (RGCs) are the major component of retinal nervous tissue. It plays a key role in conducing visual signal by feeling, conducting and processing, and thus are the main cells enabling vision in the retina. Therefore, RGCs death is an important factor causing irreversible visual function damage in DR [7].

As an important inflammatory factor, high mobility group box 1 (HMGB-1) is expressed in all eukaryotic cells. It is a type of chromosome binding protein involved in cell growth, proliferation, differentiation, migration, and nerve growth, and is closely related to a variety of diseases, including tumors, autoimmune disease, and cardiovascular disease [8–10]. HMGB-1 may play a role in stabilizing chromosome structure and regulating transcription and translation by binding with DNA. HMGB-1 is largely released when the cell suffers pathological damage, apoptosis, or necrosis, leading to immune system activation and inflammatory damage [11,12]. It was found that HMGB-1 expression increased significantly in DR patients, which can promote angiogenesis and inducing inflammation. Thus, HMGB-1 is a leading factor in DR inflammation and participates in the DR process [13,14]. HMGB-1 as a therapeutic target for DR treatment has become an important research focus. However, whether targeting HMGB-1 can protect RGCs and delay DR occurrence and progression has not yet been determined. This study aimed to investigate the impact of HMGB-1 on RGCs by siRNA interference.

Material and Methods

Reagents and instruments

RGC-5 cells were bought from the ATCC cell bank. DMEM, EDTA, and penicillin-streptomycin were obtained from Hyclone. B27, CNTF, BDNF, enzyme, and glutamine were purchased from Sigma. Dimethyl sulfoxide and MTT were purchased from Gibco. PVDF membrane was obtained from Pall Life Sciences. Lipo2000 transfection reagent was bought from Invitrogen. Western blotting-related chemical reagents were purchased from Shanghai Beyotime Biotechnology Co., LTD. ECL reagent was obtained from Amersham Biosciences. TLR4 primary antibody and secondary antibody were obtained from Cell Signaling. HMGB-1 ELISA kit was purchased from R&D. Other reagents were purchased from Shanghai Sangon Biotechnology Co., LTD. Labsystem Version1.3.1 microplate reader was bought from Bio-Rad.

Methods

RGC-5 cell culture and grouping

RGC-5 cells were seeded in dishes at 1×10^6 cells/cm^2, and maintained in serum-free DMEM medium (containing 100 U/ml penicillin and 100 μg/ml streptomycin), together with 50 μg/L CNTF, 1: 50 B27, 40 μg/L CNTF, and 25 mmol/L glucose in a humid atmosphere containing 5% CO_2 at 37°C. The cells were randomly divided into 3 groups: a normal control group, a high glucose group (cells maintained in medium with 55 mmol/L glucose), and a siRNA HMGB-1 group.

SiRNA HMGB-1 transfection

SiRNA HMGB-1 (Shanghai Genepharma, China) were transfected into RGC-5 cells using Lipofectamine 2000 reagent according to the manufacturer's instructions. The sequence of siRNA HMGB-1 was 5'-CUAGAGUUCUCCUUGGAAA-3'.

Real-time PCR

Total RNA was extracted by Trizol and the cDNA was synthesized. The primers used are listed in Table 1. The cycling conditions consisted of an initial, single cycle of 1 min at 52°C, followed by 35 cycles of 30 s at 90°C, 50 s at 58°C, and 35 s at 72°C. Gene expression levels were quantified using an optimized comparative Ct (ΔΔCt) value method.

ELISA

An ELISA kit was used to detect HMGB-1 expression changes according to the manual. Major steps included: placing 50 μl of diluted standard product into the corresponding reaction holes to prepare the standard curve and adding 50-μl samples to each hole. After washing the plates 5 times, 50 μl of enzyme reagent was added. The plates were washed again 5 times after being incubated at 37°C for 30 min. We inserted 100 μl of color agent into each hole and the plates were incubated at 37°C for 15 min. The reaction was terminated after adding 50 μl of termination liquid. The plates were measured at 450 nm wavelength to get the absorbance value (OD value). The sample concentration was calculated according to the OD value and standard curve.

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Table 1. Primers.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
GADPH	AGTGCCAGCCTCGTCTCATAG	CGTTGAACTTGCCGTGGGTAG
HMGB-1	CTCAGCGGATCTAAACGGAAT	CACATTCTGGCGCTCCGTA
TLR4	TCAGAAACTGCTCGGTCAGA	GCCTCAGGGGATTAAAGCTC
NF-κB	GATCCAGGATGAGGGGATTT	CAGGGTGTCTCCTGGTCTGT

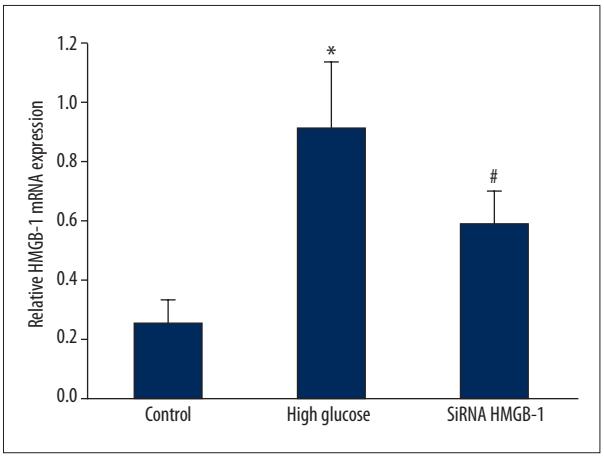

Figure 1. HMGB-1 mRNA expression in RGC-5 cells. * P=0.015, compared with control; # P=0.032, compared with high glucose group.

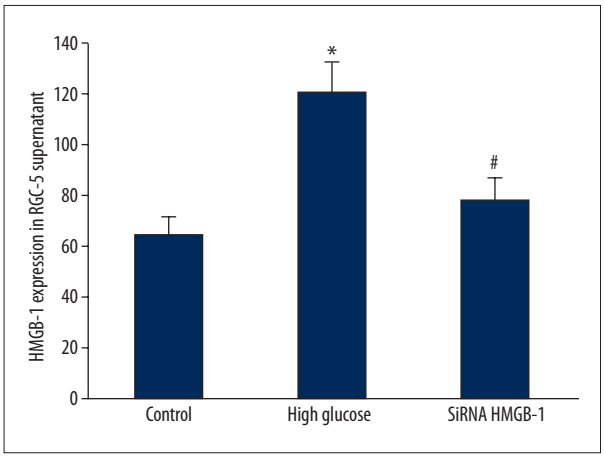

Figure 2. HMGB-1 expression in the supernatant.* P=0.022, compared with control; # P=0.029, compared with high glucose group.

MTT

Cells were seeded into 96-well plates at a density of 3×10^3 cells/well and incubated overnight at 37°C. After addition of 20 μL of MTT solution to each well, plates were incubated for 4 h at 37°C and 150 μL of DMSO was added. Absorbance of each well at 570 nm was read using a spectrophotometer.

Western blot

The cells were digested with lysis buffer. Total protein was separated by denaturing 10% SDS – polyacrylamide gel electrophoresis. After being incubated with TLR4 and NF-κB primary antibodies, the PVDF membrane was detected with chemiluminescence and calculated with Quantity One.

Statistical analysis

All statistical analyses were performed using SPSS16.0 software. Numerical data are presented as means and standard deviation ($\bar{\chi}\pm S$). Differences between multiple groups were analyzed using 1-way ANOVA. P<0.05 was considered as a significant difference.

Results

HMGB-1 mRNA expression in RGC-5

Real-time PCR was applied to detect HMGB-1 mRNA expression in RGC-5. We found that HMGB-1 mRNA was significantly overexpressed in RGC-5 under the high glucose environment (P=0.015). SiRNA HMGB-1 transfection obviously inhibited high glucose-induced HMGB-1 mRNA up-regulation (P=0.032) (Figure 1).

HMGB-1 expression in the supernatant

ELISA was performed to test HMGB-1 expression changes in the supernatant. HMGB-1 expression was 65.12±7.28 ng/ml in the normal RGC-5 cells, while it obviously increased to 121.27±11.25 ng/ml under the high glucose environment (P=0.022). SiRNA HMGB-1 transfection can markedly decrease HMGB-1 expression in the supernatant of high glucose cells, which was 78.35±9.36 ng/ml (P=0.029) (Figure 2). It was revealed that high glucose could induce HMGB-1 expression in RGC-5 cells and supernatant, whereas siRNA HMGB-1 transfection can inhibit HMGB-1 expression and secretion under the high glucose environment.

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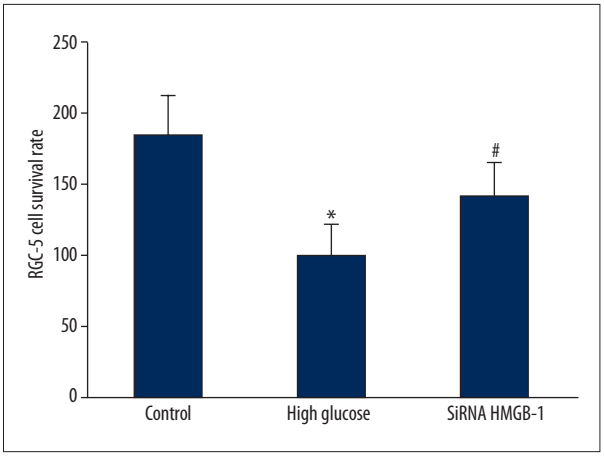

Figure 3. SiRNA HMGB-1 impact on RGC-5 cell survival. * *P*=0.026, compared with control; # *P*=0.037, compared with high glucose group.

SiRNA HMGB-1 impact on RGC-5 cell survival

MTT assay was used to determine HMGB-1 impact on RGC-5 cell survival. RGC-5 cell survival rate decreased significantly under high glucose (*P*=0.026). SiRNA HMGB-1 transfection clearly increased cell survival rate under the high glucose environment (*P*=0.037) (Figure 3). We showed that HMGB-1 overexpression under high glucose can significantly inhibit RGCs growth, while siRNA HMGB-1 can increase cell survival rate.

SiRNA HMGB-1 effect on TLR4 and NF-κB mRNA expression in RGC-5

Real-time PCR was performed to test siRNA HMGB-1 effect on TLR-4 and NF-κB mRNA expression in RGC-5. TLR-4 and NF-κB mRNA expression increased under high glucose (*P*=0.009; *P*=0.017), and they could be inhibited by siRNA HMGB-1 (*P*=0.033; *P*=0.024) (Figure 4).

SiRNA HMGB-1 effect on TLR4 and NF-κB protein expression in RGC-5

Western blotting was further used to detect siRNA HMGB-1 impact on TLR-4 and NF-κB protein expression. Similar to the mRNA results, TLR-4 and NF-κB protein were overexpressed under high glucose (*P*=0.041 and *P*=0.024, respectively), and they were inhibited by siRNA HMGB-1 transfection (*P*=0.032 and *P*=0.027, respectively) (Figure 5). We found that the high glucose environment promotes HMGB-1 expression and facilitates TLR-4 and NF-κB expression, thus activating inflammation and damaging RGCs. SiRNA HMGB-1 down-regulate TLR-4 and NF-κB mRNA and protein expression.

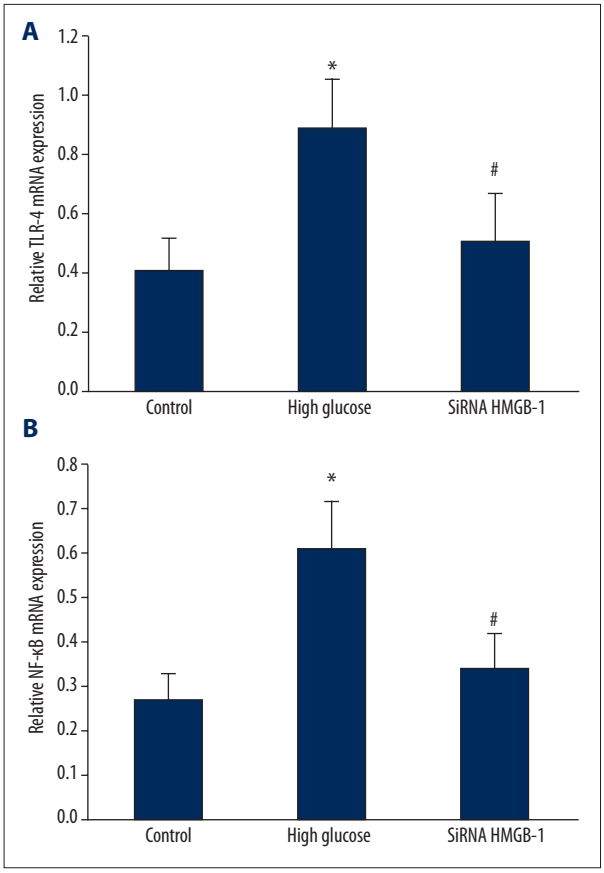

Figure 4. SiRNA HMGB-1 effect on TLR4 and NF-κB mRNA expression in RGC-5. (**A**) SiRNA HMGB-1 effect on TLR4 mRNA expression in RGC-5; (**B**) SiRNA HMGB-1 effect on NF-κB mRNA expression in RGC-5.* TLR-4 P=0.009; NF-κB P=0.017, compared with control; # TLR-4 P=0.033; NF-κB P=0.024, compared with high glucose group.

Discussion

DR is a common DM microvascular complication that leads to retinal microvascular progressive damage and seriously affects patients' physical and mental health [15]. About one-third of the diabetic patients develop diabetic retinopathy complications, mainly diabetic macular edema (DME) or proliferative diabetic retinopathy (PDR), which lead to serious visual impairment and even blindness. Patients who have had diabetes for 20 to 25 years show different degrees of DR [16]. The retina is composed of blood vessels and neurons. Optic nerve functional lesions and damage first occur in DR [5,6]. RGCs account for most of the neurons in the entire retina nerve tissue. As the earliest-differentiated neurons in the retina, RGCs mainly act on visual signal processing, conducting, and processing. However, because of its special structural features, RGC axons are easily damaged in the disease, leading to irreversible retinal damage [17]. Therefore, protecting retinal ganglia may delay DR progression [18].

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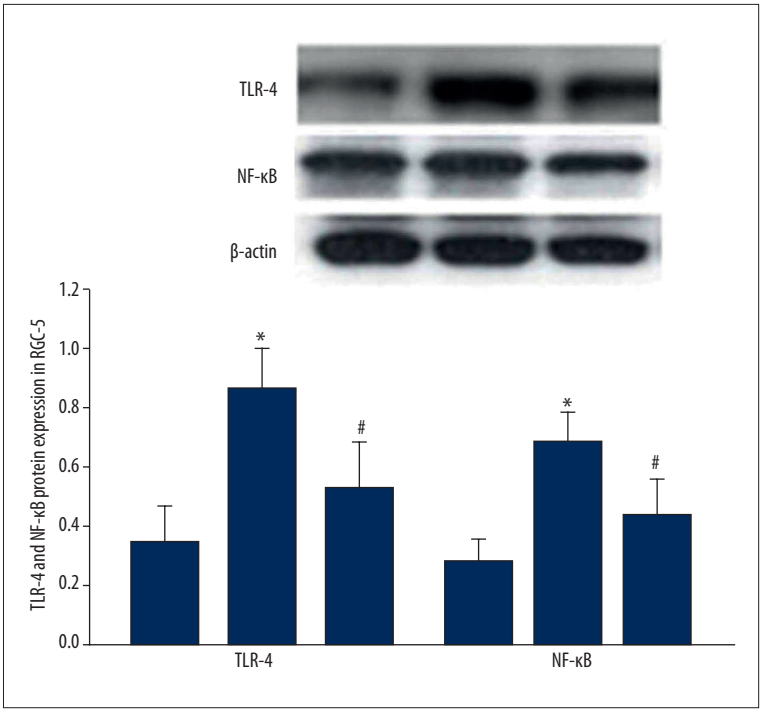

Figure 5. SiRNA HMGB-1 effect on TLR4 and NF-κB protein expression in RGC-5.* TLR-4 *P*=0.041; NF-κB *P*=0.024, compared with control; # TLR-4 *P*=0.032; NF-κB *P*=0.027, compared with high glucose group.

HGMB-1 is a type of inflammatory factor in late-stage DR and has been confirmed to be closely related to DR occurrence and development [19, 20]. DR is an inflammatory disease, and HMGB-1 expression elevated significantly in DR patients. It is the leading factor in DR inflammatory changes by promoting angiogenesis and including inflammation [13,14]. In a diabetic retinopathy rat model, we can detect HGMB-1 overexpression, as well as finding elevated receptors for advanced glycation end-products (RAGE) and elevated NF-κB level. It can further increase the retinal vascular permeability, cause inflammatory factor and adhesion molecule secretion, and destroy retinal structure and function in DR rats [20]. However, targeting HGMB-1 as a DR treatment had not been investigated. Our study shows that the high glucose environment may inhibit RGC-5 cells survival and growth. SiRNA HGMB-1 transfection can suppress high glucose-induced HMGB-1 mRNA expression and secretion in the supernatant. It can further promote cell growth under high glucose.

TLR-4 is an important receptor of HGMB-1 that is largely expressed in the nervous system. As an important member of TLRs, TLR-4 is mainly expressed in cortical neurons, dorsal root ganglion, and trigeminal neurons. Therefore, TLR-4 can regulate neuron growth and proliferation. HMGB-1 combined with TLR-4 can activate intracellular signaling pathways and the downstream signal factor NF-κB, which transfer the signal to the nucleus. This promotes inflammatory cytokines, chemokines, and colony stimulating factor expression and release, leading to leukocyte adhesion and immune cells maturation and migration, which provide conditions for inflammation outbreak [20–22]. Thus, we further focused on HGMB-1 as the therapeutic target in DR.

Conclusions

Our results confirm that the high glucose environment could promote HMGB-1 expression and activate its downstream TLR-4 and NF-κB overexpression. It can further activate inflammation and cause retinal ganglion cells damage, while siRNA HMGB-1 can down-regulate TLR-4 and NF-κB mRNA and protein expression.

Most importantly, targeting HMGB-1 can inhibit inflammation, promote RGCs survival, and thus delay DR progress through blocking the HMGB-1-TLR4-NF-κB signaling pathway.

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