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Long non-coding RNA TUG1 inhibits apoptosis and inflammatory response in LPS-treated H9c2 cells by down-regulation of miR-29b

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ABSTRACT

Objective: Myocarditis is an important cause for cardiovascular morbidity and mortality in children and adults. The lncRNA taurine up-regulated gene 1 (TUG1) plays important roles in cell apoptosis and inflammation in tumor and liver injury. The present study aimed to investigate the role of TUG1 in LPS-injured H9c2 cells and explore the underlying molecular mechanism.
Methods: H9c2 cells were stimulated with LPS to induce inflammatory injury. The expression of TUG1 was altered by transient transfections. Cell viability and apoptotic cell rates were detected by CCK-8 assay and flow cytometry assay, respectively. Inflammatory response was determined by detecting levels of inflammatory cytokines using qRT-PCR and ELISA. Furthermore, western blot analysis was conducted to assess the expression levels of core factors related with apoptosis and activations of NF-κB and JAK/STAT signaling pathways.
Results: LPS exposure reduced cell viability but enhanced cell apoptosis and inflammation in H9c2 cells. Moreover, TUG1 expression was down-regulated in LPS-injured H9c2 cells. TUG1 overexpression attenuated LPS-induced injuries in H9c2 cells, evidenced by augmented cell viability, declined apoptotic cell rates and decreased levels of pro-apoptotic factors and inflammatory cytokines. Inversely, TUG1 inhibition exerted the opposite effects. More importantly, TUG1 negatively modulated the expression of miR-29b and miR-29b mimic blocked the effect of TUG1 overexpression on cell viability, apoptosis, inflammation and inactivation of NF-κB and JAK/STAT signaling pathways in LPS-stimulated H9c2 cells.
Conclusion: This study demonstrated that TUG1 played the anti-apoptotic and anti-inflammatory roles in LPS-injured H9c2 cells via down-regulating miR-29b and inhibiting NF-κB and JAK/STAT pathways.

1. Introduction

Myocarditis, an inflammatory disease of the muscular walls of the heart, is one of the most common causes of a pediatric dilated cardiomyopathy. Myocarditis can also lead to heart failure and sudden death in young adults [1,2]. Many viruses associated with common childhood viral infections have been reported to be involved in myocarditis [2]. It makes difficult to establish a consensus on diagnosis and management to the pediatric myocarditis owing to the broad spectrum of presentation, lack of clear diagnostic criteria, and practice variation [3]. Increasing evidences have revealed that inflammation acted as a critical role in myocarditis and dilated cardiomyopathy [4,5], resulting in a focus of considerable research on the anti-inflammatory therapy to treat myocarditis.

Long noncoding RNAs (LncRNAs) are a class of noncoding RNAs that are longer than 200 nucleotides in length with limited protein-coding potential [6]. More recently, a number of lncRNAs have been reported to play crucial roles in the pathological process of many human diseases, such as cancers [7,8], central nervous system diseases [9,10], cardiovascular diseases [11–13], and liver ischemia reperfusion injury [14,15]. The lncRNA taurine up-regulated gene 1 (TUG1), which was originally detected in a genomic screen in taurine-treated mouse retinal cells, has been reported to be highly conserved in mammals. Recent studies show that TUG1 is involved in the regulation of apoptosis and proliferation in many human tumor cells [16,17]. However, the role of TUG1 in cardiomyocytes has not been fully explored yet.

The present study aimed to explore the function of TUG1 in LPS-induced cell apoptosis and inflammation response of H9c2 cells as well as the basic molecular mechanisms of TUG1. We found that TUG1 exerted the anti-apoptotic and anti-inflammatory roles in LPS-injured H9c2 cells. The regulatory mechanism might partially through the down-regulation of miR-29b caused by TUG1.

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2. Materials and methods

2.1. Cell culture and LPS treatment

H9c2 cardiomyoblasts were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA), which containing 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies Corporation, Carlsbad, CA, USA). The cells were incubated at 37 °C in an atmosphere of 95% air and 5% CO_2. For inducing inflammatory injury, cells were treated by LPS at the concentration of 10 μg/ml for 12 h.

2.2. Cell transfection

Short-hairpin RNA directed against human lncRNA TUG1 was ligated into the U6/GFP/Neo plasmid (GenePharma, Shanghai, China), which was referred as to sh-TUG1. The full-length TUG1 sequences were constructed in pEX-2. And it was referred as to pEX-TUG1. The lipofectamine 3000 reagent (Life Technologies Corporation, Carlsbad, CA, USA) was used in cell transfection assay according to the manufacturer's instructions. The plasmid carrying a non-targeting sequence was used as a negative control (NC) of sh-TUG1 that was referred as to sh-NC. For analyzing the function of miR-29b, miR-29b mimics and the corresponding negative control (NC) were synthesized (Life Technologies Corporation) and transfected into cells in the study. Since the highest transfection efficiency was obtained at 48 h, thus 72 h post-transfection was considered as the harvest time in the subsequent experiments.

2.3. Quantitative real time RT-PCR analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. 6 μg of total RNA from all samples were reversely transcribed into cDNA using a Reverse Transcription Kit (Takara, Dalian, China). The expression levels of TUG1, IL-6, IL-8 and TNF-α were determined by qRT-PCR using the SYBR Green Master Mix (Takara). As for expression level of miR-29b, total RNA was reversely transcribed with Taqman MicroRNA Reverse Transcription Kit and the followed real-time PCR was performed with Taqman MicroRNA Assay (both from Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction. GAPDH was used for the normalization of mRNA and TUG1. U6 was used for the normalization of miR-29b. The results were presented as fold changes relative to U6 or GAPDH and were calculated using the $2^{-\triangle\triangle CT}$ method.

2.4. CCK-8 assay

Cells were seeded in 96-well plate with 5000 cells/well, and cell viability was assessed by Cell Counting Kit-8 assay (CCK-8; Dojindo Molecular Technologies, Gaithersburg, MD). Briefly, after the cells were transfected and stimulated, the CCK-8 solution was added to the culture medium, and the cultures were incubated at 37 °C in humidified 95% air and 5% CO_2 for 1 h. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

2.5. Apoptosis assay

Cell apoptosis analysis was conducted using propidium iodide (PI) and fluorescein isothiocynate (FITC)-conjugated Annexin V staining method. Briefly, cells were rinsed in phosphatebuffered saline (PBS) and then were stained in PI and FITC-Annexin V from a FITC Annexin V/PI kit (BD Biosciences, San Diego, CA, USA) in the presence of 50 μg/ml RNase A (Sigma-Aldrich). After incubation for 1 h at room temperature in the dark, cell apoptosis was detected using a FACS can (Beckman Coulter, Fullerton, CA, USA). The data were analyzed by using FlowJo software (TreeStar, Ashland, OR, USA).

2.6. ELISA

Culture supernatant was collected and concentrations of inflammatory cytokines (IL-6, IL-8 and TNF-α) were assessed by enzyme-linked immunosorbent assay (ELISA) according to protocols supplied by the manufacturer (R&D Systems, Abingdon, UK). The levels were normalized to cell protein concentrations.

2.7. Western blot

The protein used for western blotting was extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Basel, Switzerland). The samples were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Bis-Tris Gel system (Bio-Rad) was established for western blotting according to the manufacturer's instructions. All the blots were transfected onto the polyvinylidene Difluoride (PVDF) membranes (Millipore, Billerica, MA) and then were blocked in 5% BSA at room temperature for 1 h. Primary antibodies: Caspase 3 (sc56053), cleaved Caspase 3 (sc22171), Cytochrome C (Cyto. C, sc13156), p65 (sc56735), p-p65 (sc136548), IκBα (sc1643), p-IκBα (sc8404; Santa Cruz Biotechnology, USA), as well as p-JAK (#74,129), JAK (#3344), p-STAT3 (#9145), STAT3 (#4904), and β-actin (#4970; Cell Signaling Technology, Beverly, MA, USA) were prepared in 5% blocking buffer at a dilution of 1:1000. The membrane was incubated with primary antibodies at 4 °C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase (1:5000; Sigma-Aldrich) for 1 h at room temperature. After rinsing, the PVDF membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 μl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

2.8. RNA immunoprecipitation (RIP)

Anti-MS2 RIP and anti-AGO2 RIP were performed as previously described by using the EZ4magna RNA Immunoprecipitation Kit (Millipore, USA) [18].

2.9. Statistical analysis

All experiments were repeated at least three times. All data of three independent experiments were presented as the mean ± SD. Statistical analysis were performed by using GraphPad Prism 6 software (GraphPad Software, USA). The *P*-values were calculated using a one-way analysis of variance (ANOVA) for more than two groups, or two-tailed Student's *t*-test between two groups. $P < 0.05$ was considered as statistically significant difference.

3. Results

3.1. LPS exposure induced cell apoptosis and inflammation in H9c2 cells

LPS stimulation usually induces inflammation and cell apoptosis. As shown in Fig. 1A, CCK-8 assay result revealed that LPS treatment significantly reduced viability of H9c2 cells ($P < 0.01$). Then, we assessed cell apoptosis and inflammation in LPS-injured H9c2 cells. As shown in Fig. 1B, the apoptotic cell rate was obviously elevated in LPS-treated H9c2 cells compared with the control group ($P < 0.01$). Correspondingly, LPS treatment induced the increased expressions of cleaved-Caspase 3 and Cyto.C (Fig. 1C). After incubation with LPS, expressions

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