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Long non-coding RNA TUG1 protects renal tubular epithelial cells against injury induced by lipopolysaccharide via regulating microRNA-223

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ABSTRACT

Background: Lupus nephritis (LN) is a serious complication of systemic lupus erythematosus (SLE). Long non-coding RNA taurine upregulated gene 1 (lncRNA TUG1) exerted critical regulatory effects on inhibiting cell injury and inflammation. However, its role in LN is still unclear.

Methods: HK-2 cells were treated with lipopolysaccharide (LPS) to simulate cell inflammatory injury. Cell viability and apoptosis, as well as pro-inflammatory factors expression were measured, respectively. Then, HK-2 cells were transfected with pEX-TUG1 or sh-TUG1 to explore the effects of TUG1 on LPS-induced cell injury. Potential binding effects between TUG1 and microRNA-223 (miR-223), as well as between miR-223 and Sirtuin 1 (Sirt1) were verified. miR-223 mimic or miR-223 inhibitor was transfected to assess the effects of miR-223 on cell injury. Finally, the roles of Sirt1 in LPS-induced HK-2 cell injury and activation of phosphatidylinositol 3-kinase/protein kinase 3 (PI3K/AKT) and nuclear factor kappa B (NF-κB) pathways were explored.

Results: LPS administration inhibited HK-2 cell viability and proliferation, increased expression of pro-inflammatory factors, and promoted cell apoptosis. TUG1 overexpression protected HK-2 cells against LPS-induced injury via negatively regulating miR-223 expression. TUG1 suppression had opposite effects. Sirt1 was a direct target gene of miR-223 in HK-2 cells, which participated in the effects of miR-223 on HK-2 cells and was related with the activation of PI3K/AKT and NF-κB pathways.

Conclusion: TUG1 protected HK-2 cells against LPS-induced inflammatory injury by regulating miR-223 and Sirt1 expression, and then activating PI3K/AKT and inactivating NF-κB pathways. TUG1 might be a potential therapeutic target for LN treatment.

1. Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by production of autoantibodies and immune complex precipitation [1]. The accumulation of immune complex precipitation will triggering multiple organ inflammation and tissue damage, including heart, joints, skin, blood vessel, nervous system, lung and liver, as well as kidney [2]. SLE is a heterogeneous disease which predominantly affects young females and the young females account for about 90% of all SLE patients [3]. The kidney damage is the most common complication in the visceral injury by SLE. Lupus nephritis (LN), one of the most frequent and serious manifestations of SLE, is the major cause of substantial morbidity and mortality or as a result of immunosuppressive drug toxicity. About 50%–80% of SLE patients accompany with LN [4,5]. Therefore, researches about the mechanism of renal injury caused by SLE will be helpful for the treatment of LN.

Long non-coding RNAs (lncRNAs) is a novel class of non-coding RNAs with more than 200 nucleotides (nt) in length and without protein-coding potential [6]. After the completion of Human Genome Project (HGP), lncRNAs are considered as the "junk sequences" during evolution [7]. Currently, increasing number of researches proved that lncRNAs were involved in the regulation of multiple cellular biological functions via modulating gene expression [8]. The aberrantly expression of lncRNAs was found in a variety of cancer cells and some lncRNAs were identified as the diagnostic and therapeutic indicators [9–11]. Previous study has revealed that lncRNAs played a key role in the regulation of immunological functions and autoimmunity [12]. For example, lncRNA cyp2c91 has been found to exert regulatory effects on perturbations in immune system [13]. Previous experiment also suggested that lncRNA NEAT1 was a novel inflammatory regulator in human lupus [14]. However, the roles of lncRNAs in the SLE remain unclear yet.

LncRNA taurine upregulated gene 1 (TUG1) is a newly discovered lncRNA, which was proved to be associated with cancer and mainly

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expressed in retinal and brain tissues [15–17]. Researchers found that TUG1 was related with lung cancer and might promote lung cancer cell proliferation [18]. Khalil et al. proved that TUG1 was a major regulator of cell cycle in transcriptional response to DNA damage [19]. In addition, TUG1 has been proved to protect mouse livers against cold-induced injury via inhibiting apoptosis and inflammation [20]. However, as far as we know, the effects of TUG1 on renal tubular epithelial cell inflammatory injury have not been studied.

In this study, we explored the roles of TUG1 in lipopolysaccharide (LPS)-simulated LN cell inflammatory injury model. The potential regulatory effects between TUG1 and microRNAs (miRNAs), as well as between miRNA and target gene were also investigated. With the deep understanding about the role of TUG1 in LN, our researches might provide a possible therapeutic target for LN and SLE treatment.

2. Materials and methods

2.1. Cell culture and treatment

The human renal proximal tubule epithelial cells (hRPTECs) HK-2 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM-F12, 3:1, Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) in a humidified incubator (Thermo Fisher Scientific, Waltham, MA, USA) with 5% CO_2 at 37 °C. The culture medium was changed every 3 days until confluence was achieved.

The stable cultured cells at passage 10 or below were seeded into 6-well plates (Thermo Fisher Scientific, Waltham, MA, USA) at a density of 5×10^4 cells/well and cultured in humidified incubator. When cells reached to 80% confluence, the culture medium was replaced by serum-free DMEM medium and cells were cultured for another 24 h. Then, the cells were treated with 1, 5, or 10 μg/ml of *E.coli*-derived LPS (026:B6, Sigma-Aldrich, St, Louis, MO, USA) for 4 h to simulate inflammatory response [21]. Cells without LPS induction were used as negative control.

2.2. Cell viability assay

Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD, USA) assay was used to assess cell viability. Briefly, cells were seeded into 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) with 5×10^3 cells/well and pre-incubated for 24 h. After stimulation and/or corresponding administrations, 10 μl CCK-8 solution was added into the culture medium of each well, and the plates were incubated for another 1 h at 37 °C. Then, the absorbance of each well at 450 nm was measured by Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA).

2.3. Cell proliferation assay

5-btomo-2'-deoxyuridine (BrdU) incorporation assay (Beyotime Biotechnology, Shanghai, China) was conducted to detect the proliferation of HK-2 cells after corresponding administrations. Briefly, cells were seeded into 6-well plates with 5×10^4 cells/well. BrdU (1 mg/ml) was added into each well of the plate before relevant treatment by 4 h. After that, BrdU positive (+) cells in each group were counted under Microscope (Nikon, Japan). Cell proliferation of each group was quantified by number of BrdU positive (+) cells/number of total cells.

2.4. Apoptosis assay

Cells apoptosis analysis was performed using Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China) with flow cytometry assay. Briefily, 24 h after corresponding administrations, HK-2 cells were collected, washed twice with cold phosphate buffered saline (PBS, Sigma-Aldrich) and re-suspended in Annexin-binding buffer. After incubation for 30 min, cells were stained with Annexin V-FITC and PI in the presence of 50 μg/ml RNase A (Sigma-Aldrich) for another 30 min in the dark at room temperature. Then, flow cytometry analysis with FACScan (Beckman Coulter, Fullerton, CA, USA) was conducted to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive). The data were analyzed using FlowJo software 10 (TreeStar, Ashland, OR, USA).

2.5. Cell transfection

Short-hairpin RNAs (shRNAs) directed against human TUG1 or Sirtuin 1 (Sirt1) were ligated into U6/GFP/Neo plasmid (GenePharma, Shanghai, China), respectively, and referred as sh-TUG1 or sh-Sirt1. The full-length of TUG1 or Sirt1 sequences was constructed into pEX-2 plasmids (GenePharma), respectively, and referred as pEX-TUG1 or pEX-Sirt1. The plasmid carrying a non-targeting sequence was used as a negative control (NC) of sh-TUG1 and sh-Sirt1 that was referred as shNC. Empty pEX-2 plasmid was acted as NC of pEX-TUG1 and pEX-Sirt1 that was referred as pEX. miR-223 mimic, miR-223 inhibitor and their respective NC (Scramble and inhibitor control) were synthesized by Life Technologies Corporation (Carlsbad, CA, USA) and transfected into cells, respectively. All transfections were performed by Lipofectamine 3000 reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The stably transfected cells were selected by culture medium containing 0.5 mg/ml G418 (Sigma-Aldrich, St Louis, MO, USA). The sequence of sh-TUG1 was: 5'-GGTGGTTGAAAGGAATCCT-3'. The sequence of shNC was: 5'-CCTAAGGTTAAGTCGCCCTC-3'. The sequences of miR-223 mimic were: 5'-CGUGUAUUUGACAAGCUGAGUU-3' (sense) and 5'-CUCAGC UUGUCAAAUACACGUU-3' (antisense). The sequence of miR-223 inhibitor was: 5'-AACUCAGCUUGUCAAAUACACG-3'. The sequence of Scramble was: 5'-AAGCGCGCUUUGUAGGAUUCG-3'. The sequence of inhibitor control was: 5'-CAGUACUUUUGUGUAGUACAA-3'.

2.6. RNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from HK-2 cells after corresponding administrations using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The purity of RNA was confirmed by the ratio of optical densities at 260 nm/280 nm. The One Step SYBR® PrimeScript® PLUS RT-PCR Kit (TaKaRa Biotechnology, Dalian, China) was used for the Real-Time PCR analysis to test the expression level of TUG1. The extracted RNA was reversed by using TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, USA), and TaqMan Universal Master Mix II supplemented with the TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA, USA) were used for qPCR to measure the expression levels of miR-223. For Sirt1 measurement, reverse transcription was performed by using the Multiscribe™ Reverse transcription Kit (Applied Biosystems, CA, USA) supplemented with random hexamers or oligo (dT). The qPCR was performed by using FastSTART Universal SYBR Green Master (ROX) (Roche, USA) on the ABI PRISM 7500 real-time PCR System (Applied Biosystems, Foster City, CA). All primers were obtained from the NCBI GeneBank database and were synthesized by GenePharma (Shanghai, China). The primer sequences were: TUG1 forward 5'-CTGAAGAAAGGCAACATC-3', TUG1 reverse 5'-GTAGGCTA CTACAGGATTTG-3'; miR-223 forward 5'-CAGCACAAAAGGAAACTCA CCC-3', miR-223 reverse 5'-GAGCCTGGGGACTTTCCAC-3'; Sirt1 forward: 5'-AAAGGAATTGGTTCATTTATCAGAG-3' Sirt1 reverse: 5'-TTG TGGTTTTTCTTCCACACA-3'. GAPDH forward: 5'-GCACCGTCAAGGCT GAGAAC-3', GAPDH reverse: 5'-TGGTGAAGACGCCAGTGGA-3'; U6 snRNA forward: 5'-CTCGCTTCGGCACGACATATACT-3', U6 snRNA reverse: 5'-ACGCTTCACGAATTTGCGTGTC-3'. Data were analyzed