miR-297 Protects Human Umbilical Vein Endothelial Cells against LPS-Induced Inflammatory Response and Apoptosis

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Key Words
miR-297 • Inflammation • Apoptosis • HUVECs

Abstract

Background/Aims: Recently, microRNA-297 (miR-297) and signal transducer and activator of transcription 3 (STAT3) have been demonstrated to be involved in dysfunction of vascular endothelial cells and inflammatory conditions, such as sepsis. The present study aimed to investigate the role of miR-297 and STAT3 in lipopolysaccharide (LPS)-induced inflammatory human umbilical vein endothelial cells (HUVECs). Methods: HUVECs were stimulated by different concentrations of LPS. miR-297 mimics were transfected into HUVECs to over-express miR-297. Western blot was used to detect the expressions of STAT3, inflammatory cytokines, adhesion molecules and apoptosis-related proteins. Results: Compared with parental HUVECs, the expression of miR-297 was significantly down-regulated, while the expression of STAT3 was obviously up-regulated in LPS-induced HUVECs. The expressions of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin were also increased in LPS-induced HUVECs than those in parental HUVECs. In addition, LPS induced apoptosis of HUVECs through up-regulation of Bax and cleaved caspase 3 expressions. Conversely, miR-297 mimics inhibited LPS-activated expressions of STAT3, inflammatory cytokines, and adhesion molecules, and protected HUVECs against LPS-induced apoptosis through inhibition of Bax and cleaved caspase 3 expressions. Mechanistically, the 3′-untranslated region (3′-UTR) of STAT3 mRNA was validated as a direct target of miR-297. Over-expression of STAT3 partially abrogated protective effects of miR-297, whereas silencing of STAT3 contributed to miR-297-mediated biological effects. Conclusion: miR-297 protects HUVECs against LPS-induced inflammatory response and apoptosis by targeting STAT3 pathway. Thus, miR-297 may be a promising therapeutic target for patients with sepsis.
Introduction

Sepsis is characterized by acute inflammatory responses to exogenous bacteria and multiple organ injury [1, 2], which is one of the leading causes of death among all hospitalized patients in the worldwide. The inflammatory response helps to eliminate pathogens and necrotic cell fragments, and promotes wound healing and tissue regeneration. However, excessive inflammatory mediators would aggravate tissue damage [3, 4]. Severe sepsis or septic shock can trigger damage to numerous organs, including brain, heart, kidney, liver, and lung [5-7]. Therefore, it is crucial to elucidate the molecular mechanisms underlying inflammatory injury in sepsis.

MicroRNAs (miRNAs), a kind of non-coding small molecule RNA, are involved in regulation of post-transcriptional gene expressions [8, 9], which play a pivotal role in physiological conditions and inflammatory diseases [10-12]. Accumulating evidence suggests that the expression of miR-297 is frequently altered in many tumors and inflammatory diseases, such as bacterial infections or sepsis [13-17]. In addition, abnormal activation and dysfunction of endothelial cells are required for various vascular inflammatory diseases, such as sepsis, diabetes, rheumatoid arthritis, and inflammatory bowel diseases [18-21]. During inflammation, the activated vascular endothelium expresses various adhesion molecules (VCAM-1, ICAM-1 and E-selectin), which can recruit leukocytes, and regulate leukocyte attachment on the vascular endothelial cells at the early stage [21]. However, the role of miR-297 in inflammatory responses of vascular endothelial cells in sepsis is still unclear.

In the present study, we hypothesized that miR-297 influences the expressions of inflammatory cytokines in LPS-stimulated HUVECs, and is involved in the apoptosis of HUVECs. Firstly, we established a model of inflammatory HUVECs using LPS-induced HUVECs in vitro. Then, we over-expressed miR-297 by transfection of miR-297 mimics into HUVECs. Finally, we investigated the role of miR-297 in LPS-induced inflammatory response and apoptosis using in-vitro assays.

Materials and Methods

Cell culture and ethics statement

This study was approved by the Ethics Committee of Shandong Provincial Third Hospital. Written informed consent was obtained from all subjects prior to the study. HUVECs were obtained from five human donors. Umbilical cords (10 cm in length) were resected soon after birth, and immediately stored in phosphate buffered saline (PBS). The HUVECs were obtained from the lumens of human umbilical veins using collagen enzyme solution. Then, cells were cultured in endothelial cell medium (ECM) (ScienCell, United States). HUVECs were incubated at 37°C in 5% CO2 and maintained using standard cell culture. After reaching a confluence of 80%, HUVECs were detached using 0.25% trypsin-EDTA. Human embryonic kidney-293T (HEK-293T) cells (Cell Resource Center of Shanghai Institute of Life Science, Shanghai, China) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), 100 U/mL penicillin (Invitrogen) and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified and 5% CO2 atmosphere.

Cell transfection

Negative control miRNA (miR-NC), miR-297 mimics, STAT3-small interfering RNA (si-STAT3), and negative control siRNAs (si-control) were all purchased from GenePharma Co. (Shanghai, China). The full-length STAT3 was ligated into a pcDNA3.1 plasmid (Shanghai GenePharma Co., Ltd, China), and the recombinant plasmid was referred to as pcDNA3.1-STAT3. Lipofectamine 2000 regent (Invitrogen, Carlsbad, CA, USA) was utilized for cell transfection in line with the protocol of the manufacturer. The empty pcDNA3.1 plasmid was also transfected into cells, termed as a vector group. Stable transfection was selected by 0.5 mg/mL G418 (Sigma-Aldrich, St. Louis, MO, USA). Cells transiently transfected with miRNAs were harvested at 72 h post-transfection.
**Western blot**

Briefly, cells were lysed with RIPA lysis buffer in the presence of protease inhibitor cocktail (Merck, Darmstadt, Germany). Cell debris was removed by centrifugation at 12,000 rpm for 12 min. The protein content was determined with BCA kit and separated by 8~10% SDS-PAGE, and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked in 5% (w/v) non-fat dry milk in Tris-buffered saline (TBS) and 0.1% (v/v) Tween 20 for 1 h at room temperature, and then incubated with the primary antibodies (1:500; Abcam, Cambridge, MA, USA) at 4 °C overnight. GAPDH (1:1000; Santa Cruz Biotechnology, Paso Robles, CA, USA) was used as an internal control. After incubation of appropriate secondary horseradish peroxidase-conjugated antibodies including HRP-conjugated anti-rabbit or anti-mouse (1:1000; Cell Signaling Technology, Inc., Beverly, MA, USA) for 1 h, blots were visualized with ECL kit. Band intensities were measured by densitometry (Chemi Doc Documentation System/Quantity One quantitation software, Bio-Rad Laboratories Inc, Hercules, CA, USA). Variations in protein levels were expressed as fold change compared with control after normalization to GAPDH.

**Quantitative real-time PCR and RT-PCR**

Total RNA was isolated from cells using the TRizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. An aliquot of 2 μg RNAs were reversely transcribed with a PrimeScript RT reagent kit (Bio-Rad Laboratories, Hercules, CA, USA), and real-time PCR was performed using SYBR Green PCR Master Mix (Bio-Rad Laboratories) on a MyiQ Single Color Real-time PCR Detection System (Bio-Rad Laboratories). The sequence-specific primers for the indicated genes were synthesized by Sangon Biotech (Shanghai, China). To detect miRNA expression, reverse transcription was performed using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) and PCR reactions were conducted using the TaqMan® MicroRNA Assay Kit (Applied Biosystems). Primers for miR-297, and the small nuclear RNA U6 were from RiboBio Co., Ltd. (Guangzhou, China). The fold change in expression for each gene was calculated using the 2−ΔΔCT method, with U6 as an internal control. After amplification, 10 μl of products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining under UV light.

**Apoptosis assay**

Quantization of apoptotic cells were performed using Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, China). In brief, the transfected cells were planted in 6-well plates at a density of 1×10⁶ cells per well and were subjected to 100 ng/mL LPS for 24 h. Cells were then collected and resuspended in 200 μL binding buffer (Beijing Biosea Biotechnology, China) containing 10 μL Annexin V-FITC. After 30 min incubation in the dark at room temperature, 5 μL PI and 300 μL binding buffer were added, and the samples were immediately analyzed on a flow cytometer (BD Biosciences, USA). Annexin-V positive cells were recognized as apoptotic cells.

**MiRNA target prediction**

The potential target genes of miRNAs were predicted in silico using different miRNA target prediction algorithms: TargetScan (http://www.targetscan.org/), DIANA-MICROT (http://diana.cslab.ece.ntua.gr/micro-CDS/), miRDB (http://mirdb.org/miRDB/), miRanda (http://www.microrna.org/microrna/), and miRwalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/).

**Dual luciferase reporter assay**

Vectors containing wild-type (Wt) or mutant (Mut) STAT3 3′-UTR were obtained from GenePharma (Shanghai, China) and co-transfected with miR-297 mimics into HEK-293T cells. Forty-eight hours later, HEK-293T cells were lysed. Firefly and Renilla luciferase activities were measured with a Dual-Luciferase Reporter Assay Kit (TransGen Biotech Co., Ltd. China) using a dual luciferase-reporter assay system (Berthold, Germany). The results were evaluated by normalizing the firefly luciferase activity to the Renilla luciferase activity.
Statistical analysis
Data are presented as means ± standard deviation (SD). Comparisons among multiple groups were carried out using the one-way analysis variance (ANOVA) followed by Bonferroni’s comparison test. Statistical calculations were performed by GraphPad Prism (GraphPad Software, San Diego, CA, USA). P < 0.05 was considered statistically significant.

Results

The inflammatory HUVECs model is successfully established by LPS
To generate LPS-induced inflammatory HUVECs model, we firstly explored the rational dosing and the time course of LPS action. HUVECs were administrated with different concentrations of LPS (0, 10, 100, or 1000 ng/mL) at different time points (0, 6, 12, 24, and 48 h). Then we measured the expression level of miR-297 using RT-PCR and qRT-PCR. Meanwhile, inflammatory cytokines (IL-6 and TNF-α) and adhesion molecules (ICAM-1, VCAM-1, and E-selectin) were further detected by Western blot analysis. The results showed that miR-297 expression was significantly down-regulated after LPS treatment in a time- and dose-dependent manner (P < 0.001, Fig. 1A). In addition, the protein expression levels of STAT3, IL-6, TNF-α, ICAM-1, VCAM-1, and E-selectin were markedly increased in a time- and dose-dependent manner upon LPS stimulation (P < 0.001, Fig. 1A). Notably, when HUVECs were subjected to 100 ng/mL LPS for 24 h, miR-297 expression decreased by approximately 50%, while the expressions of inflammatory cytokines and adhesion molecules increased by approximately 56%. These findings indicated the inflammatory HUVECs model was successfully established after stimulation for 24 h with 100 ng/mL LPS.

LPS induces apoptosis of HUVECs
To figure out the role of LPS in apoptosis of HUVECs, AnnexinV-FITC/PI staining was used to detect apoptosis, and Western blot analysis was used to measure the expression levels of apoptosis-related proteins in inflammatory HUVECs model. We found that the percentage of apoptotic cells was markedly elevated, accompanied with the increase of LPS concentration (Fig. 1B), indicating cell viability was severely affected. Compared with HUVECs treated with 0 ng/mL LPS, the difference of cell apoptosis in LPS-induced HUVECs was statistically significant (P < 0.001). Similarly, Bcl-2 expression was significantly down-regulated, while the expressions of Bax and cleaved caspase-3 were gradually up-regulated with the increase of LPS concentration (P < 0.001, Fig. 1C). These findings suggested that LPS induced apoptosis of inflammatory HUVECs.

miR-297 mimics increase miR-297 expression in HUVECs
In this work, we further investigated how miR-297 affected cell apoptosis and expressions of inflammatory cytokines. Because miR-297 was down-regulated in LPS-induced HUVECs, miR-297 mimics were transfected into HUVECs to increase the expression of miR-297. Then, HUVECs were administrated in the presence or absence of 100 ng/mL LPS for 24 h. The qRT-PCR analysis revealed that miR-297 expression in HUVECs was markedly up-regulated after transfection with miR-297 mimics compared with miR-NC (P < 0.001, Fig. 2A).

LPS-induced inflammatory injury is alleviated by miR-297 over-expression
Firstly, we investigated the effect of miR-297 over-expression on the expressions of inflammatory cytokines. HUVECs with miR-297 mimics were administrated in the presence or absence of 100 ng/mL LPS for 24 h. Expressions of inflammatory cytokines were detected by Western blot assay. We found that miR-297 over-expression obviously inhibited LPS-induced expressions of STAT3, IL-6, TNF-α, ICAM-1, VCAM-1 and E-selectin (P < 0.001, Fig. 2B). At the same time, negative associations were confirmed among miR-297 and inflammatory cytokines, suggesting miR-297 may play an important role in suppressing inflammatory responses of LPS-induced HUVECs. Additionally, apoptosis assay showed that
the percentage of apoptotic cells of HUVECs was significantly increased at 24 h following stimulation with 100 ng/mL LPS. By contrast, the percentage of apoptotic cells of HUVECs transfected with miR-297 mimics was significantly decreased (P < 0.01, Fig. 2C), indicating that miR-297 had a protective effect on HUVECs. Finally, we demonstrated that Bax and cleaved caspase-3 proteins were highly expressed in LPS-induced HUVECs, which were subsequently suppressed by miR-297 mimics (P < 0.001, Fig. 2D). However, Bcl-2 protein level was reduced by LPS, which was subsequently increased after transfection with miR-297 mimics (P < 0.001, Fig. 2D). These results suggested miR-297 protected HUVECs against LPS-induced inflammatory injury.
STAT3 is a direct target of miR-297

TargetScan and miRanda algorithms predicted that the transcriptional STAT3 was a candidate target of miR-297. Then we performed Western blot and dual luciferase reporter assay to identify whether the 3'-UTR sequences of STAT3 mRNA were targeted by miR-297. Two plasmids were generated by cloning the respective fragments from the 3'-UTR of STAT3 into the downstream region of the Renilla luciferase reporter gene. HEK-293T cells were then co-transfected with one of the wild type or mutant reporter vectors and miR-297 mimics or miR-NC. Western blot analysis revealed that the expression of STAT3 protein was obviously down-regulated in HEK-293T cells co-transfected with wild-type STAT3 3'-UTR and miR-297 mimics (P < 0.001, Fig. 3A and B). The dual luciferase reporter assay validated that the luciferase activity of the wild-type was inhibited by miR-297 mimics as compared with miR-NC (P < 0.01, Fig. 3C), while there were no significant differences in the luciferase activity of the mutant type between miR-297 mimics and miR-NC group (P > 0.05, Fig. 3D). These results indicated that STAT3 was a direct target of miR-297.
LPS-induced inflammatory injury is exacerbated by restored STAT3 expression

In order to explore the effect of STAT3 on LPS-induced inflammatory injury in HUVECs, the pcDNA3.1-STAT3 plasmid or pcDNA3.1 vector were co-transfected into HUVECs with miR-297 mimics. The percentage of apoptotic cells was determined by flow cytometry. The effects of STAT3 on inflammatory cytokines and adhesion molecules in LPS-induced HUVECs were evaluated by Western blot analysis. The results revealed the protein levels of IL-6, TNF-α, ICAM-1, VCAM-1 and E-selectin in STAT3-overexpressed HUVECs were drastically up-regulated compared with their respective controls (P < 0.01, Fig. 4A). Over-expression of STAT3 restored the expressions of miR-297 mimics-inhibited STAT3, IL-6, TNF-α, ICAM-1, VCAM-1 and E-selectin. In addition, the percentage of apoptotic cells was significantly increased in STAT3-overexpressed HUVECs than that in LPS alone-treated group. Over-expression of STAT3 markedly increased miR-297 mimics-repressed apoptotic rate (P < 0.001, Fig. 4B). To further confirm our results at a molecular level, we performed Western blot to detect the protein expression levels of apoptosis-related biomarkers, including Bax, Bcl-2 and cleaved caspase-3. As illustrated in Fig. 4C, the expressions of Bax and cleaved caspase-3 proteins in STAT3-overexpressed HUVECs were obviously up-regulated compared with LPS alone-treated group, whereas this promotion effect was rapidly inhibited by co-transfection with miR-297 mimics (P < 0.001). In addition, the expression level of Bcl-2...
was down-regulated by STAT3 over-expression and subsequently up-regulated after co-transfection with miR-297 mimics. These data indicated that LPS-induced inflammatory injury was exacerbated by restored STAT3 expression.

Suppression of STAT3 contributes to the protective effects of miR-297 on HUVECs

To confirm if miR-297 exert its biological functions by targeting STAT3, we co-transfected si-STAT3 and miR-297 mimics into HUVECs. Then HUVECs were administrated with 100 ng/mL LPS for 24 hours. Firstly we demonstrated that STAT3 protein level was indeed decreased by si-STAT3 compared with si-control (P < 0.001, Fig. 5A). Then, Annexin V-FITC/PI apoptosis assay revealed that cell apoptosis was significantly alleviated by co-transfection of si-STAT3 and miR-297 mimics, compared with three other groups (P < 0.001), indicating cell viability may be restored by suppression of STAT3. Further, co-transfection of si-STAT3 and miR-297 mimics obviously inhibited the expressions of inflammatory cytokines and adhesion molecules compared with si-STAT3 alone or miR-297 mimics alone (P < 0.001, Fig. 5A). Finally, we identified that co-transfection of si-STAT3 and miR-297 mimics obviously inhibited the expressions of Bax and cleaved caspase-3, and markedly up-regulated the
expression of Bcl-2 compared with three other groups (P < 0.001, Fig. 5B). Our findings indicated that suppression of STAT3 contributed to the protective effects of miR-297 on HUVECs.

Discussion

To date, it has been reported that activation of endothelial cells and exposure to endotoxins lead to the development of sepsis. LPS, also known as lipoglycans and endotoxins, is released from bacterial membranes, which binds to the endothelial cell surface, and triggers a potent inflammatory responses \[22, 23\]. Recently, miRNAs have been demonstrated to play a crucial role in LPS-mediated inflammatory factors. Although miR-297 is associated with LPS-induced inflammatory processes, and involved in the development of sepsis \[24\], it is not clear how miR-297 modulates inflammatory responses in LPS-induced vascular endothelial cells.

In previous studies, LPS can activate STAT3 signaling via protein phosphorylation \[23\]. Once activated, phosphorylated STAT3 dimerizes and enters the nucleus to activate transcription of various genes \[25\]. Currently, STAT3 signaling has been shown to be activated in inflammatory vascular endothelial cells, and induced the expressions of inflammatory cytokines \[26-28\]. Once STAT3 is persistently activated, septic diseases will occur \[29\]. Till now, STAT3-involved pathway in sepsis is still unknown. In this work, we explored the potential molecular mechanisms of miR-297 and STAT3 using LPS-induced inflammatory HUVECs model. We found that miR-297 over-expression decreased expressions of LPS-induced STAT3, inflammatory cytokines and adhesion molecules, and inhibited LPS-induced apoptosis of HUVECs. Besides, apoptosis-related proteins induced by LPS were also reversed by miR-297 over-expression. Considering that STAT3 is closely related to T and B cell activation, immune responses and inflammatory reaction \[30\], we assumed that miR-297 may protect HUVECs against LPS-induced inflammatory response and apoptosis through inhibition of STAT3.

MiRNAs achieve their biological function through binding the 3'-UTR of target genes and regulating the expression levels of protein-coding genes. Recent studies showed that some miRNAs participated in regulation of STAT3 pathway. Luo Y et al. reported that miR-
20b negatively regulated VEGF expression by targeting STAT3 in hepatocellular carcinoma cells [31]. Cheng DL et al. suggested that miR-34a promoted iNOS secretion from pulmonary macrophages in septic suckling rats through activating STAT3 pathway [32]. Zhang W et al. indicated that overexpression of miR-4443 promoted the resistance of non-small cell lung cancer cells to epirubicin by targeting INPP4A, and regulating activation of JAK2/STAT3 pathway [33]. In this study, we demonstrated that miR-297 reduced the expression of STAT3 protein by targeting its 3’-UTR. Meanwhile, miR-297-inhibited inflammatory cytokines, adhesion molecules and apoptosis were further repressed by silencing of STAT3. On the contrary, LPS-induced cell injury was exacerbated by restored STAT3 expression. These findings suggested that LPS/miR-297/STAT3 pathways may play a crucial role in inflammatory processes of HUVECs.

**Conclusion**

In conclusion, our study suggested that miR-297 inhibited LPS-induced inflammatory response and apoptosis of HUVECs by regulating STAT3 signaling pathway. These findings may help us understand the pathogenesis of sepsis, and provide a novel therapeutic approach for prevention and/or treatment of sepsis.

**Acknowledgements**

We are thankful to doctors and nurses in the ICU for their great support.

**Disclosure Statement**

The authors have declared that no conflict of interest exists.

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