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microRNA-211-3p has a Role in the Effects of Lipopolysaccharide on Endoplasmic Reticulum Stress in Cultured Human Skin Fibroblasts

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Data Interpretation D
Manuscript Preparation E
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Background: Lipopolysaccharide (LPS) in bacterial infection of skin wounds delays wound healing. This study aimed to investigate the effects of LPS and endoplasmic reticulum stress in cultured skin fibroblasts and microRNA-211-3p (miR-211-3p) signaling.

Material/Methods: Human skin fibroblasts were cultured in increasing concentrations of LPS at 0 ng/ml, 5 ng/ml, 10 ng/ml, and 20 ng/ml for 0, 12 h, 24 h, 36 h, and 48 h. Cell proliferation was determined using the MTT assay. Protein expression levels of the transcription factors GRP78, CHOP, p-JNK, and the endoplasmic reticulum stress apoptosis proteins, caspase-12 and Bcl-2, were determined by Western blot. The expression of miR-211-3p in human skin fibroblasts was detected by quantitative polymerase chain reaction (qPCR).

Results: Cell proliferation of human skin fibroblasts decreased with increasing concentrations of LPS in a dose-dependent and time-dependent way. Protein levels of GRP78, CHOP, p-JNK, caspase-12, and Bcl-2 were increased 8 h and 12 h after LPS treatment compared with 0 h and 4 h after treatment. However, the expression of miR-211-3p was decreased in human skin fibroblasts after treatment with LPS. When miR-211-3p was overexpressed, the endoplasmic reticulum stress/CHOP related proteins, including GRP78, CHOP, p-JNK, caspase-12, and Bcl-2 were unchanged after the addition of LPS. Overexpression of miR-211-3p also reduced inhibitory effects of LPS on the growth of human skin fibroblasts.

Conclusions: This study showed that microRNA-211-3p had a role in the effects of LPS on endoplasmic reticulum stress and CHOP activation in cultured human skin fibroblasts.

MeSH Keywords: Endoplasmic Reticulum Stress • Lipopolysaccharides • MicroRNAs

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Background

In skin wounds, bacterial contamination usually comes from the soil, clothes, and skin and can include Clostridium sp. [1]. In the early stage of skin injury, many types of bacteria can be cultured in the wound. After a period of natural competition and treatment with antibiotics, bacterial infection may persist [2]. Bacterial infection can result in a significant increase in plasma levels of lipopolysaccharide (LPS) [3,4].

The expression of endoplasmic reticulum stress signaling components, including CHOP, are significantly associated with autophagy [5,6]. Endoplasmic reticulum stress functions include a reduction in protein translation and the expression of endoplasmic reticulum chaperones, such as immunoglobulin heavy chain binding protein (BiP/GRP78) [7]. The expression of CHOP induces apoptosis via endoplasmic reticulum stress inducers [6,8].

MicroRNAs (miRNAs) regulate many genes, including BCL-2 [9,10]. Skin wounds are commonly infected with bacteria, and infection is associated with high levels of LPS. Therefore, this study aimed to investigate the effects of LPS and endoplasmic reticulum stress in cultured skin fibroblasts and microRNA-211-3p (miR-211-3p) signaling.

Material and Methods

Reagents

Unless otherwise specified, all chemical reagents were purchased from the Sigma-Aldrich (St. Louis, MO, USA). All antibodies, including GRP78 (No. 3177), CHOP (No. 5554), IgG (No. 14708), GAPDH (No. 5174), caspase-12 (No. 2202), phospho-JNK (No. 4668), and Bcl-2 (No. 4223) were purchased from Cell Signaling Technology (Danvers, MA, USA). The human skin fibroblast cell line was obtained from ScienCell (Carlsbad, CA, USA). Human skin fibroblasts were cultured at 37°C with 5% CO_2 in humidified air.

Cell growth rate assay

The cell counting kit 8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) was used to determine cell viability and cell proliferation. Briefly, cells were seeded into 96-well plates at a density of 5×10^3 per well, and different concentrations of lipopolysaccharide (LPS) (0 ng/ml, 5 ng/ml, 10 ng/ml, and 20 ng/ml) were added. Cells were collected at different time points (12 h, 24 h, 36 h, and 48 h). CCK-8 solution, 10 µL CCK-8 in 100 µL medium, was added, and the cells were incubated for 1 h. The final step was to extract 0.1 mL of culture medium, for analysis using an enzyme-linked immunosorbent assay (ELISA) at 450 nm. Each test was repeated five times. The growth rate of the cells was calculated as follows:

% growth rate=(mean experimental absorbance/mean control absorbance) ×100

Quantitative polymerase chain reaction (qPCR)

Total cellular microRNA (miRNA) was obtained using Tiangen reagent. MiRNA complementary DNA (cDNA) was synthesized using M-MLV reverse transcriptase, and qPCR was performed using SYBR Premix Ex Taq [11].

Western blot

Proteins from the supernatants of cultured human skin fibroblasts were separated with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes for immunoblotting. The membranes were blocked and then incubated with primary antibodies overnight at 4°C. The membranes were washed three times with phosphate-buffered saline (PBS) and incubated with species-compatible peroxidase-conjugated secondary antibodies. Immunoreactive bands were detected using ECL reagents (Pierce Biotechnology, Rockford, IL, USA).

Statistical analysis

Statistically significant differences in gene expression levels between treatment groups were determined using one-way analysis of variance (ANOVA), followed by Newman-Keuls post hoc analysis. Data were presented as the mean ± standard deviation (SD).

Results

Effect of lipopolysaccharide (LPS) on the growth of human skin fibroblasts

The effects of LPS on human skin fibroblasts growth are shown in Figure 1. The growth rates of human skin fibroblasts decreased with increasing concentrations of LPS and with increasing time. The maximum decrease in growth was at an LPS concentration of 20 ng/ml at 24 h.

Effect of LPS on the endoplasmic reticulum stress pathway in human skin fibroblasts

To determine the effects of LPS on the endoplasmic reticulum stress pathway, protein levels for GRP78, CHOP, p-JNK, caspase-12, and Bcl-2 were measured at 0, 4, 8, and 12 h after LPS (20 ng/ml) treatment. Levels of GRP78, CHOP, p-JNK, caspase-12, and Bcl-2 were significantly increased at 8 h and 12 h after LPS (20 ng/ml) treatment compared with at 0 and 4 h after treatment ($P<0.05$) (Figure 2A, 2B). The expression of microRNA-221-3p

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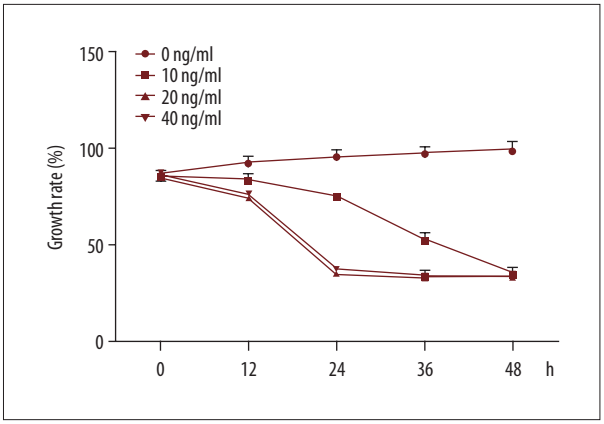

Figure 1. Growth rates of human skin fibroblasts following treatment with lipopolysaccharide (LPS). Human skin fibroblasts treated with increasing concentrations of lipopolysaccharide (LPS), 0 ng/ml, 5 ng/ml, 10 ng/ml, and 20 ng/ml for 0, 12 h, 24 h, 36 h, and 48 h.

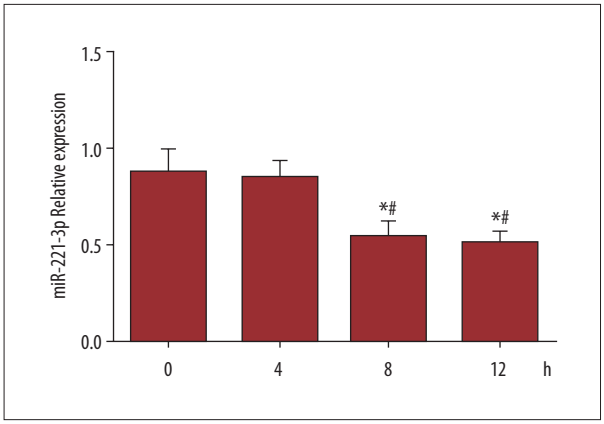

Figure 3. Effect of lipopolysaccharide (LPS) (20 ng/ml) on microRNA-221-3p (miR-221-3p) in human skin fibroblasts determined by quantitative polymerase chain reaction (qPCR).

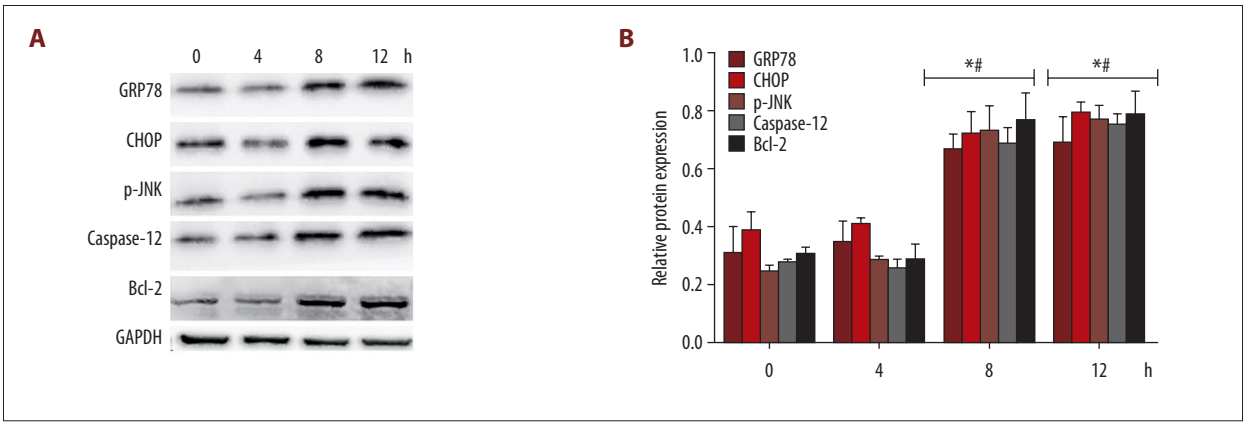

Figure 2. Effect of lipopolysaccharide (LPS) (20 ng/ml) on the endoplasmic reticulum stress pathway in human skin fibroblasts. (**A**) Representative Western blots showing levels of GRP78, CHOP, p-JNK, caspase-12, Bcl-2, and GAPDH. (**B**) Histograms summarizing the results shown in (**A**). Results are expressed as the mean ± standard deviation (SD) (n=5). * Significantly different *vs.* 0 h ($p<0.05$); # significantly different *vs.* 4h ($p<0.05$).

(miR-221-3p) in human skin fibroblasts was detected by quantitative polymerase chain reaction (qPCR). Expression levels of miR-211-3p significantly decreased at 8 h and 12 h after LPS treatment compared with 0 and 4 h after treatment (Figure 3).

Effect of LPS on endoplasmic reticulum stress after miR-211-3p overexpression

To further elucidate the effects of miR-211-3p, miR-211-3p was overexpressed. The results showed that the expression GRP78, CHOP, p-JNK, caspase-12, and Bcl-2 was unchanged after LPS treatment when miR-211-3p was overexpressed (Figure 4). Overexpression of miR-211-3p reduced the inhibitory effects of LPS (20 ng/ml) on the proliferation of human skin fibroblasts, and cell growth was significantly increased when miR-221-3p was overexpressed (Figure 5).

Discussion

In skin injury, bacterial infection can lead to chronic inflammation and promotes angiogenesis. Lipopolysaccharide (LPS) is widely used in experimental models of wound healing and infection [12,13]. LPS can stimulate inflammation and induce chronic inflammation [14]. In malignancy, LPS treatment can induce tumor cell proliferation and facilitate cell migration and has been shown to promote angiogenesis [14]. In this study, the effect of LPS on skin fibroblasts was investigated. LPS is the main component of Gram-negative bacteria [15]. In this study, LPS inhibited the growth of human skin fibroblasts and was associated with impaired function of endoplasmic reticulum and expression of markers of apoptosis, which occurs *in vivo* to remove damaged cells. In the present study, the optimal concentration of LPS was 20 ng/mL.

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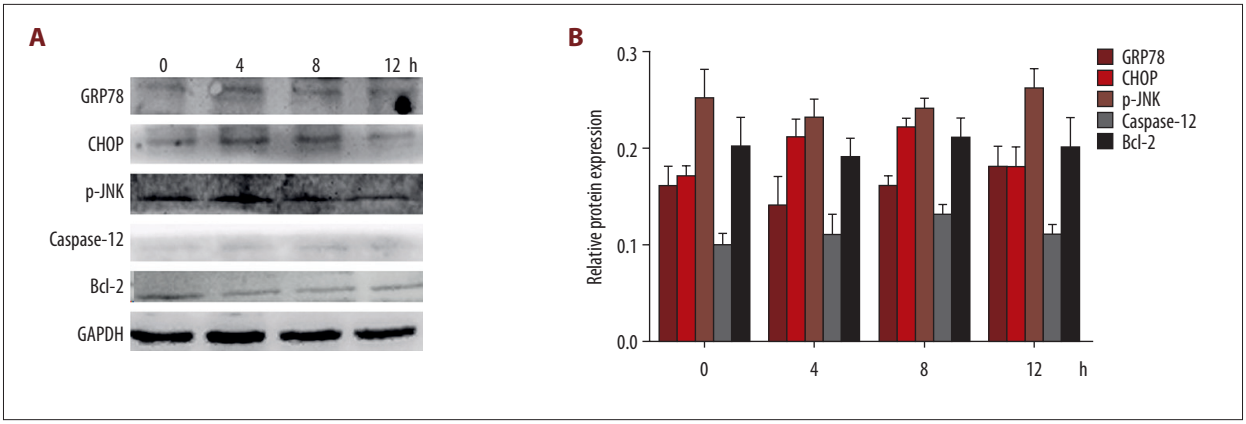

Figure 4. Effect of lipopolysaccharide (LPS) on endoplasmic reticulum stress following overexpression of microRNA-221-3p (miR-221-3p). (**A**) Representative Western blots showing levels of GRP78, CHOP, p-JNK, caspase-12, Bcl-2, and GAPDH. (**B**) Histograms summarize the results shown in (**A**). Results are expressed as the mean ± standard deviation (SD) (n=5).

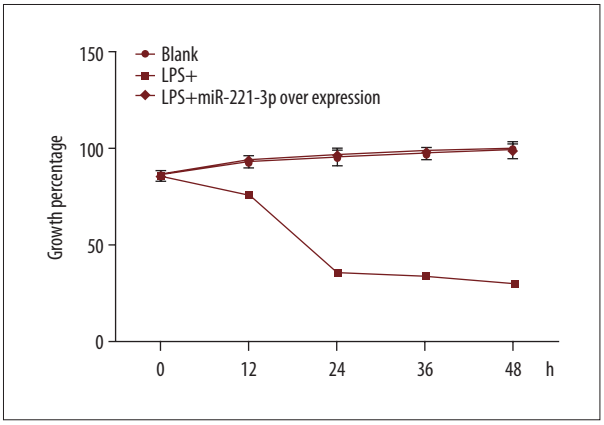

Figure 5. Percentage of human skin fibroblast growth after overexpression of microRNA-221-3p (miR-221-3p).

MicroRNAs (miRNAs) are non-coding single-stranded RNAs containing approximately 21 to 24 nucleotides, which regulate gene expression by base-pairing of 2 to 8 nucleotides with the 50-untranslated or 30-untranslated regions of target mRNAs, primarily in the cytoplasm [16,17]. miRNAs are involved in many biological processes, including tumorigenesis and endoplasmic reticulum stress. A previous study showed the microRNA-221-3p (miR-221-3p) regulated endoplasmic reticulum stress in myeloma cells [18]. In this study, miR-211-3p suppressed the inhibitory effects of LPS on the growth of human skin fibroblasts. Also, when miR-211-3p was overexpressed, endoplasmic reticulum stress/CHOP related proteins, including GRP78, CHOP, p-JNK, caspase-12, and Bcl-2 were unchanged in response to LPS, in contrast to the effects of LPS alone.

Conclusions

This study aimed to investigate the effects of LPS and endoplasmic reticulum stress in cultured skin fibroblasts and microRNA-211-3p (miR-211-3p) signaling. The findings showed that microRNA-211-3p had a role in the effects of LPS on endoplasmic reticulum stress and CHOP activation in cultured human skin fibroblasts.

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