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Expression and Immune Effect of Toll-Like Receptor 4 in Human Trophoblast Cells

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Summary：This study investigated the expression and immune effect of TLR4 in human trophoblast cells. The expression level of TLR4 mRNA in normal and LPS-stimulated human term trophoblast cells (1 mg/L LPS, 12 h) was detected by RT-PCR. In LPS-stimulated human term trophoblast cells of TLR4-blocked group and non-TLR4-blocked group, and normal term trophoblast cells of blank control group, apoptosis rate was measured by flow cytometry (FCM), and the level of TNF-α determined by using enzyme linked immunosorbent assay (ELISA) respectively. RT-PCR results showed that the expression level of TLR4 mRNA in LPS-stimulated human trophoblast cells was significantly higher than that in normal cells (P<0.01). FCM revealed that there was significant difference in apoptosis rate of LPS-stimulated human term trophoblast cells between TLR4-blocked group and non-TLR4-blocked group (P<0.05), or between TLR4 antibody-blocked group and blank control group. ELISA indicated that the level of TNF-α in LPS-stimulated human trophoblast cells also had statistical differences between TLR4 antibody-blocked group and non-TLR4 antibody-blocked group (P<0.05). Our results suggest that TLR4 plays an important role in the immunological mechanism of apoptosis and secretion of TNF-α of human term trophoblast cells stimulated by LPS.
Key words：Toll like receptor 4; lipopolysaccharide; human trophoblast cells; apoptosis; TNF-α

The human maternal-fetal interface is immunologically unique in that it has both innate and acquired immunological mechanism that can tolerate embryo implantation and permit fetal development while simultaneously respond to a broad diversity of invasive pathogens. In order to accommodate these disparate functions, it must rely upon coordinately immunological mechanism[1]. Toll-like receptors (TLRs) are kinds of pattern receptors which have evolved in innate mechanism that can recognize specific structures produced by microorganisms called pathogen-associated molecular patterns (PAMPs), activate the acute inflammatory response, and help to set up acquired immunological mechanism[2, 3]. It has been demonstrated that lipopolysaccharide (LPS), a lipid A component of cell wall of gram-negative pathogenic bacteria, has immunological stimulatory properties because it is one of the best-known PAMPs, and its principal signaling receptor is TLR4[4-6].

The aim of this study was to characterize the expression of TLR-4 in normal and LPS-stimulated human term trophoblast cells, investigate the relationship between the expression of TLR-4 and apoptosis rate or secretion of TNF-α, and explore the immune effect of TLR4 on human trophoblast cells stimulated by LPS.

1 MATERIALS AND METHODS

1.1 Materials

Trizol reagent was purchased from Gibco Co. (USA). LPS was purchased from Linfei Co. (China). M-MLV reverse transcriptase and Taq DNA polymerase were purchased from Promega Co. (USA). Antibody against TLR4 was purchased from Bioscience Co. (USA). ELISA reagent kit for human TNF-α was purchased from Jingmei Co. (China). Annexin V-FITC cell apoptosis reagent kit was purchased from Kaiji Co. (China). PCR primers were synthesized and purified by Jingmei Co. (China).

1.2 Isolation and Culture of Trophoblast Cells

Trophoblast tissues were obtained from women of normal pregnancy subjected to elective terminations by cesarean section performed at Union Hospital, Tongji Medical College, HUST (China), washed with 4°C pre-cold D-Hanks solution, and then cut into small pieces. Cells were scraped from the membranes, transferred to 0.25% trypsin+0.02% EDTA digestion buffer, and incubated for 10 min with shaking. An equal volume of DMEM/F12 medium containing 10% FBS was added to inactivate the trypsin, then the mixture was eluted and centrifuged at 1000 r/min for 10 min, and the supernatant was discarded. The collected pellets were re-suspended in DMEM/F12 medium, mixed with the same volume of Lymphocyte Separation Media (3:2, v:v), transferred to a 15-mL tube and centrifuged at 2000 r/min for 25 min. In the end, the cellular interface which was cloudy and grey-white was the purified trophoblast cells. The cells were then re-suspended in DMEM/F12 medium containing 10% FBS and adjusted to a density of 2×10^6/mL, and cultured in an incubator with 5% CO_2 at 37°C.

1.3 Experiment Grouping

Cells were seeded into 24-well plates (2×10^4/well), cultured overnight until they reached 80%–90% conflu-

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ence, and divided into three groups. In TLR4-blocked group, the cells were incubated with anti-TLR4 antibody (10 μg/mL) for 2 h, and then with LPS (1 mg/L) for another 12 h. In non-TLR4-blocked group, the cells were incubated with LPS (1 mg/L) alone for 12 h. The normal trophoblast cells served as blank controls. After incubation, the cells in each group were digested with trypsin, and centrifuged at 1200 r/m for 8 min. The cell supernatant and pellets were collected. Some cell pellets in non-TLR4-blocked and blank control groups were treated with 1-mL Trizol reagent, and total RNA was isolated for real-time PCR. The remaining cell pellets in non-TLR4-blocked and blank control groups and the cell pellets in TLR4-blocked group were subjected to flow cytometry for the detection of apoptosis. The supernatant in each group was stored at –20°C for later ELISA.

1.4 RT-PCR

Total RNA was isolated from cell pellet in non-TLR4-blocked and blank control groups using Trizol reagent according to the manufacturer's instructions. A total of 200-ng RNA was reversely transcribed to cDNA. Resulting cDNA was stored at –20°C until amplification. Quantitative RT-PCR was conducted for the determination of TLR4, with β-actin serving as internal control. Primers used in the RT-PCR analysis were designed as table 1.

Table 1 Sequence of RT-PCR primers

Primers	Sequences
TLR4 Upper primer	5'-AGTGTGTGTCCGCATGA T-3'
Lower primer	5'- CCACTTGGGGTCTA AGA ACG-3'
β-actin Upper primer	5'- TCCTGTGGCATCCACGAAACT-3'
Lower primer	5'-GAAGCATTTGCGGTGGACGAT-3'

RT-PCR total reaction volume was 25 μL, and 30 cycles of PCR were performed at 95°C for 5 min, then 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and 72°C for 7 min. All RT-PCR products were subjected to 2% agarose gel eletrophoresis and photographed.

1.5 Flow Cytometry

Cell pellets in all three groups were used for measuring apoptosis rate by flow cytometry (FCM). Cell pellet were washed twice with pre-cold PBS, and then diluted in 250-μL PBS. The cells were adjusted to a density of 1×10^6/mL, incubated with 5 μL FITC-Annexic and 5-μL PI buffer at room temperature for 15 min, and then mixed with 400-μL PBS to be analyzed using a FACS-Vantage cell sorter (BD Biosciences, USA). Data were analyzed using FACSDiva software (BD Biosciences, USA).

1.6 ELISA

Cell supernatants of all three groups were used for detecting TNF-α level using a Human Cytokine Array Kit according to the manufacturer's instructions. Data were analyzed using ELISA software.

1.7 Statistical Analysis

The results were processed by SPSS 12.0 software. Data were expressed as $\bar{x}\pm s$. The statistical significance was analyzed using homogeneity test for variance first and then one-factor analysis of variance. A P value less than 0.05 was considered to be statistically significant.

2 RESULTS

2.1 RT-PCR

RT-PCR showed that the expression level of TLR4 mRNA in non-TLR4-blocked group was significantly higher than that in blank control group with the difference being statistically significant ($P<0.01$) (fig. 1).

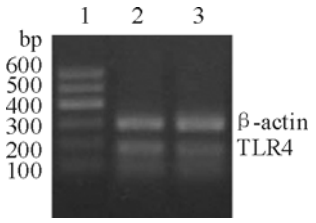

Fig. 1 Electrophoretogram of the TLR4 mRNA expression
1: Marker 2: Non-TLR-4-blocked group; 3: Blank control group

2.2 FCM Detection of Apoptosis Rate

FCM revealed that there was statistically significant difference in apoptosis rate between TLR4-blocked group and non-TLR4-blocked group, or between non-TLR4-blocked group and blank control group ($P<0.05$), suggesting that the apoptosis rate of trophoblast cells stimulated by LPS was significantly increased, but the rate was significantly decreased after TLR4 was blocked with anti-TLR4 antibody (table 2, fig. 2).

Table 2 Comparison of apoptosis rate among groups

Groups	Apoptosis rate (%)
TLR4-blocked	21.70±1.5395
Non-TLR4-blocked	47.03±11.2607*
Blank control	20.43±2.1502△

*$P<0.05$ as compared with TLR4-blocked group, △$P<0.05$ as compared with non-TLR4-blocked group

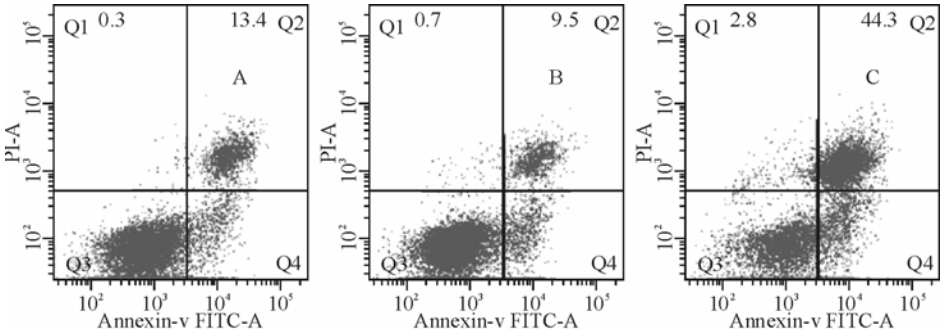

Fig. 2 Graphical spectrum of apoptosis rate of three groups as detected by FCM
1: Blank control group; 2: TLR4-blocked group; 3: Non-TLR4-blocked group

2.3 Determination of TNF-α Level by ELISA

ELISA revealed that there was statistically significant difference in TNF-α level between TLR4-blocked group and non-TLR4-blocked group, or between non-TLR4-blocked group and blank control group (P<0.05) (table 3).

Table 3 Comparison of TNF-α level among groups

Groups	TNF-α level
TLR4-blocked	2.072±0.053
Non-TLR4-blocked	5.728±0.122*
Blank control	1.981±0.0498△

*P<0.05 as compared with TLR4-blocked group, △P<0.05 as compared with non-TLR4-blocked group

3 DISCUSSION

TLRs were initially identified in fly as essential receptor for dorsoventral polarity during embryogenesis[7, 8]. Subsequent studies revealed that they also play essential roles in the innate immune response against infection[9, 10]. TLR proteins are a family of type Ⅰ transmembrane receptors which consist of an extracellular domain, a transmembrane domain and an intracellular Toll/IL-1 receptor homology domain. The extracellular domain which contains specific horseshoe-shaped structure is involved in ligand binding, and the intracellular domain triggers signaling cascade with Toll/IL-1 receptor (TIR), leading to transcriptional activation of genes encoding pro-infammatory mediators, and ultimately starting inflammatory response to infection[11, 12]. To date, 11 members of the TLR family have been identified in mammals, and TLR4 is thought to be the signaling transduction receptor for LPS[13]. It was found that LPS was the key pathogenic factor of Gram-negative bacteria which cause intrauterine infection[14], and placenta was an unique immune barrier to defend against invasive microbial pathogens during pregnancy[15]. In order to elucidate the involvement of TLR4 in signaling conduction pathway of LPS in the placenta, in this study, the expression levels of TLR4 mRNA in normal and LPS (1 mg/L for 12 h)-stimulated human term trophoblast cells were detected by RT-PCR. The results showed that TLR4 was detectable in term trophoblast cells, and the expression level of TLR4 mRNA in LPS-stimulated trophoblast cells was significantly higher than that in normal term trophoblast cells (P<0.05), which was consistent with those reported in the literature[16]. These findings demonstrated that TLR4 took part in the immune reaction of term trophoblast cells to LPS in placenta. After detection of the expression of TLR-4 by term trophoblast cells, the biological functions of TLR-4 in term trimester trophoblast cells were evaluated by FCM and ELISA to measure apoptosis rate and TNF-α expression level in all groups. The results showed that the apoptosis rate and TNF-α expression level in non-TLR4-blocked group were greatly higher than those in TLR4-blocked group and blank control group (all P<0.05). The results suggest that LPS stimulation, through receptor of TLR-4, triggered term trimester trophoblast cells to produce high levels of cytokines, including TNF-α, and in the meantime, this course might also induce apoptosis of term trophoblast cells sensitive to the cytokines, suggesting that the term plancenta might protect itself by this immunological mechanism, which induces apoptosis of trophoblast cells located in the infective position to restrain the spread of infection.

Other observation also showed that apoptosis of term trimester trophoblast cells induced by intrauterine infection might be associated with some pathological conditions such as preeclampsia, IUGR, and preterm labor, but the precise mechanisms have not yet been fully understood[17–20]. Our results indicated that TLR4 has a direct effect on the induction of apoptosis and TNF-α expression of term trimester trophoblast cells in response to LPS-induced infections, which may provide a novel alternative of prevention and treatment for some pregnancy-related complications resulting from intrauterine infection.

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